Chapter 3

Materials and Methods
3. Materials and Methods

3.1 Materials

Dulbecco’s Modified Eagle’s Medium (DMEM) and Superscript First Strand Synthesis kit were purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-prohibitin antibody and rabbit polyclonal anti-α-tubulin antibody were obtained from Neomarker (Fremont, CA). Anti-HSP70 antibody was obtained from Assay design (Ann Arbor, MI). HSP inhibitor I and Anti-Actin antibody was obtained from Calbiochem (Gibbstown, NJ). Alexa flour-Fluorescein isothiocyanate conjugated antibodies, Mitotracker® Red, Lysotracker® Red, Syto® green 11 and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR) and horse radish peroxidase conjugated secondary antibodies were procured from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Western blotting reagents and enhanced chemiluminescence kit were from Amersham Life Sciences, Inc. (Piscataway, NJ). CB-X™ protein assay kit was obtained from G-Biosciences (Maryland heights, MO). Complete protease inhibitor cocktail tablets were obtained from Roche (Basel, Switzerland). Seize Immunoprecipitation Kit and EZ-Link Sulfo-NHS-biotin were procured from Pierce (Rockford, IL). Reagents for polymerase chain reaction (PCR) like Taq polymerase and dNTP mixture were obtained from New England Biolabs (Beverly, MA). TNT® coupled rabbit reticulocyte system and pGEM-T easy vector kit were obtained from Promega Corporation (Madison, WI). Paclitaxel, Bafilomycin A1, 3 - Methyl Adenine, Monodansyl cadaverine, Phosphotidylinositol phospholipase C (PI-PLC), PKH67 green fluorescent cell linker kit for general cell membrane labeling, papain, peanut agglutinin (PNA), medium-199 (M199) and all other chemicals unless otherwise mentioned were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fetal Bovine Serum (FBS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). The pXG-GFP+2 vector was a kind gift from Dr. Stephen M. Beverley, Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO.

3.2 Methods

3.2.1 Cell Culture

3.2.1.1. In Vitro Leishmania donovani and L. major culture

*Leishmania donovani* promastigotes were grown on blood agar slants containing 1% glucose, 5.2% brain heart infusion agar extract, 6% v/v of rabbit blood and 1 mg/ml of gentamycin as antibiotic (Sudhandiran and Shaha, 2003). After three days of culture at 22°C on slants, fresh slants were streaked and cultured for regular maintenance. The cells were transferred to Medium 199 supplemented with 10% fetal calf serum. Before experiments, the cells were centrifuged at 130×g for 10 min to remove dead and agglutinated parasites; the supernatant was centrifuged at
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805xg for 10 min to pellet live cells which were then resuspended in appropriate amounts of media for experiments.

*Leishmania major* promastigotes were cultured in modified DMEM (DMEM pH 7.4, containing 5.96 gm/l HEPES, 5 mg/l hemin, 0.5 ml/l triethanolamine, 40 mg/l Tween 80, 13.36 mg/l adenine, 7.6 mg/l xanthine and 1mg/l of biopterin) supplemented with 10% fetal calf serum, the virulent cultures were injected in female Balb/c mice footpad for *in vivo* maintenance and footpad harvested after 4 weeks of infection to obtain fresh virulent cultures. *In vitro* cultured *L. major* cells were not used after 6 weeks in culture due to decrease in virulence of the parasites. All parasitic cultures were grown at 22°C unless otherwise specified.

### 3.2.1.2. *In Vivo Leishmania major* culture

Freshly obtained *Leishmania major* promastigotes from the footpads of infected mice were injected into the footpads of new adult mice for *in vivo* maintenance. The protocol described briefly, footpads of infected mice were harvested in sterile conditions after euthanizing the mice by cervical dislocation, in accordance to the regulations of Animal Ethics Committee at the National Institute of Immunology, New Delhi, India. The footpads were isolated by making lateral incisions in the skin of the footpad, the skin was removed and the callous mass of tissue formed during *L. major* infection was isolated. This callous mass was dissected into three to four pieces added to 10 ml of modified DMEM supplemented with 10% fetal bovine serum with 100 Units/ml penicillin and 100 μg/ml streptomycin as antibiotics, the cultures were incubated at 22°C for a week with regular monitoring. After 2-3 days of isolation and culturing of footpad, motile promastigotes appear in the culture. The culture after a week of incubation was centrifuged at 130xg for 10 min at 22°C to pellet the tissue pieces and mammalian cells. The supernatant containing motile promastigotes was centrifuged at 805xg for 10 min at 22°C, the pellet was resuspended in 10 ml of modified DMEM supplemented with 10% fetal bovine serum with antibiotics at cultured at 22°C. Few days after the *in-vitro* growth *L. major* cells were harvested by centrifugation at 805xg for 10 min at 22°C, the pellet was resuspended in modified DMEM, the cell density adjusted to $1 \times 10^6$ cells/50 μl of media and 50 μl of this cell suspension was injected in the footpads of new mice. The mice were monitored on a weekly basis for development of the lesion; mice having lesion size thickness above 0.8 cm were used for harvesting fresh promastigotes. Any animal during the experimental period showing extreme discomfort, injury or secondary infections at the lesion site was euthanized as per the regulations of Animal Ethics Committee.
3.2.1.3. In Vitro J774A.1 murine macrophage culture
Murine J774A.1 cells were maintained in phenol red free DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin as antibiotics. The cells were cultured at 37°C in 5% CO₂ and 95% air; the cultures were sub-cultured every three days or at attainment of 90% confluency.

3.2.1.4. In Vitro L. donovani culture for growth curve and metacyclogenesis
After three days of culture on slants, cells were flushed out in Medium 199 supplemented with 10% fetal calf serum. The cells were centrifuged at 130xg for 10 min to remove dead and agglutinated parasites; the supernatant was centrifuged at 805xg for 10 min to pellet live cells which were then resuspended in 10 ml of media. The cells were counted in Neubauer’s chamber and 1x10⁷ cells were added to each of the six TC 25 cm² flasks and the media volume was adjusted in each flask to 5ml. The flasks were incubated at 22°C and one flask was harvested each day for ascertaining the number of cells and number of metacyclics in the culture to determine the phase of growth. The remaining cells were lysed in 2x SDS lysis buffer for Western blotting.

3.2.1.5. In Vitro infection of J774A.1 murine macrophages with L. donovani cells
For In vitro infection studies; J774A.1 macrophages were plated on coverslips at the density of 1x10⁵ cells/coverslip in a 6 well culture plate or at a density of 5x10⁵ cells/well of a 6 well culture plate, the cells were allowed to rest for 8 hrs. The cells were challenged with the parasites at MOI (multiplicity of infection) of 1:10 for 6 hrs after which the excess unbound parasites were washed with phosphate buffered saline and the macrophages incubated at 37°C for 18 hrs. The cells were then stained with anti-prohibitin antibody to observe the expression of prohibitin in the transforming promastigotes.

For infection with paclitaxel treated parasites and infection with overexpressing parasites; shorter intervals were used for analysis of progression of infection, in such cases the cells were washed for removal of excess parasites stained and visualized by staining cells with Syto green 11 to visualize the parasite nucleus and macrophage nuclei. The % infectivity, number of parasite/cell and number of parasite/100 macrophages was calculated using the below given formulas;

1) % Infection = (Number of infected macrophage/ Number of total macrophage) X100
2) Number of parasite/cell = (Number of parasite/ Number of infected macrophage)
3) Number of parasites/100 macrophages (Infectivity Index) = Number of parasite/ 100 macrophages or (% infection X number of parasite/ cell)
3.2.1.6. In Vitro infection of J774A.1 murine macrophages with L. donovani cells and analysis by flow cytometry

For analysis of murine macrophage infection with labeled parasites, L. donovani cells were stained with PKH67 Green Fluorescent cell linker kit Sigma Aldrich (St. Louis, MO) as per the manufacturer's protocol. Briefly, a day before the experiment L. donovani cells were flushed from slants or pelleted from media, counted and approximately 2×10⁸ cells were used. The cells were pelleted down at 805xg for 10 min as described previously, all supernatant was removed from the pellet and 1ml of Diluent C added to the pellet and pipetted up and down several times to make a single cell suspension. In another centrifuge tube 4µl of the PKH dye (4×10⁻⁶ M) was added in 1ml of Diluent C, mixed gently and immediately the single cell suspension was added. The suspension was incubated for 4-5 min. After the incubation; 2 ml of fetal bovine serum was added to quench the reaction incubated for 5 min and then 5 ml of M199 added. The suspension was pelleted at 805xg for 10 min, the pellet resuspend in 10 ml of M199 supplemented with 10% fetal bovine serum and transfer into a new centrifugation tube, centrifuged again and the process was repeated for a total of 3 times after which the pellet was resuspended in 10 ml of M199 supplemented with 10% fetal bovine serum and incubate overnight at 22°C. These labeled parasites were used for infection using the protocol described in the previous section.

For flow cytometric analysis, the macrophages after washing the unbound parasites were dislodged from the culture plates by gently flushing them. These cells were analyzed by flow cytometry, the infected cells showed a shift in the staining of the macrophages towards higher intensities. To determine the % infection the numbers of macrophage cells that were positive for PKH label were used in comparison to the total number of cells counted. To determine the number of parasite/infected cell the below given formula was used;

Number of parasite/cell = (Mean fluorescence intensity of infected macrophages / Mean fluorescence intensity of labeled parasite)

3.2.2 Molecular Biology techniques

3.2.2.1 Total RNA isolation

Total RNA was isolated from cells using TRIzol reagent, Invitrogen (Carlsbad, CA) following the manufacturer's protocol. Briefly, 2×10⁸ cells were harvested and washed once with PBS. The cell pellet was lysed with 2 ml ice-cold TRIzol reagent. The lysate was centrifuged at 12,000xg for 10 min at 4°C to pellet down cellular debris, polysaccharides, and high molecular weight DNA. The supernatant was gently decanted into a fresh microcentrifuge tube and 200 µl of chloroform/ml of TRIzol was added and the tube was shaken vigorously for 15 s. The mixture was incubated at room temperature for 2-3 min before centrifugation at 12,000×g for 15 min at 4°C.
This resulted in the separation of the mixture into a lower organic phase and an upper aqueous phase. The aqueous phase containing the RNA was gently aspirated and transferred into a fresh microcentrifuge tube and 500µl of isopropanol/ml of TRizol reagent was added to precipitate the RNA. The mixture was centrifuged at 12,000×g for 10 min at 4°C to isolate the RNA as a pellet. The supernatant was discarded and the pellet was washed once with 70% ethanol, centrifuged and the pellet was air-dried and re-dissolved in appropriate quantity of nuclease-free water. The purity (A_260/A_280 > 1.8) and concentration (A_260 X dilution factor X 40) of the obtained RNA was determined by measuring the absorbance at 260 nm (A_260) and 280 nm (A_280).

3.2.2.2. First strand synthesis by reverse transcription
First strand synthesis of mRNA into cDNA was performed using First strand cDNA synthesis kit from Invitrogen (Carlsbad, CA) following manufacturer’s protocol. Briefly, 4 µg of total RNA was denatured at 65°C for 5 min in the presence of Oligo dT12-18 and dNTPs and incubated at 42°C for another 2 min with DTT, MgCl2, and RNaseOUT in 10 X reverse transcription buffer. 1 µL/reaction of the Superscript Reverse Transcriptase enzyme was added to the denatured RNA and incubated at 42°C for 50 min. The enzyme was denatured by heating at 70°C for 15 min. The reaction was completed by a quick high-speed centrifugation and the complementary RNA strand degraded by incubating with RNase H for 20 min at 37°C. The preparation was stored at -70°C.

3.2.2.3. Polymerase Chain Reaction
Polymerase chain reaction (PCR) was used to amplify specific nucleotide sequences from cDNA derived from L. donovani. The reaction consisted of an initial denaturation at 94°C for 5 min, followed by 20-30 cycles of denaturation at 94°C for 1 min, annealing at primer specific temperature for 45 s, and extension at 72°C for 90 s. A final extension at 72°C for 10 min was performed. The PCR products were resolved on 1-2% agarose gel containing ethidium bromide and visualized under ultraviolet illumination. The specific primers used are shown in the table below.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prohibitin</td>
<td>5’- ATGTCGAAGTTGCTGCAGAAG - 3’</td>
<td>5’- TCACTTCGACATGTCAGAAG - 3’</td>
</tr>
<tr>
<td>GFP</td>
<td>5’- CGCCTCTCTCTCTCTCTCTCTCTCT-3’</td>
<td>5’-GTTCCTCCCTGAAAGTGGAT -3’</td>
</tr>
<tr>
<td>GFP-Pro</td>
<td>5’-CACTACCAGCAGAAACACC-3’</td>
<td>5’-ATTTGCTGCTGACAGAG-3’</td>
</tr>
<tr>
<td>Actin</td>
<td>5’- ATGACATGGAGA AGATCTGGC -3’</td>
<td>5’-CTTCACGGTGCGCACAATCTCC -3’</td>
</tr>
</tbody>
</table>

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3.2.2.4. Agarose gel electrophoresis

DNA fragments were resolved on 1-2% agarose gel containing 0.5 μg/mL ethidium bromide in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.1). The samples were mixed with an equal volume of 2X loading dye containing bromophenol blue, and the samples were resolved by applying a voltage of ~5-7 V/cm. The resolved DNA fragments were visualized under ultraviolet illumination and the relative band size was determined by comparison against a DNA ladder with bands of known sizes. When required, images were acquired using a UVP Gel Documentation system.

3.2.2.5. Elution of DNA from agarose gel

To elute DNA from agarose gel, the samples were loaded on a gel casted with low melting point agarose (LMP agarose). The samples were resolved and visualized under UV transilluminator, and the band of interest was excised quickly using a scalpel blade. The volume of gel slice was quantitated by weighing and the DNA eluted using MinElute Gel Extraction kit, Qiagen (Hilden, Germany) as per manufacturer's protocol. Briefly, the gel was solubilized by incubating it with buffer QG at 50°C for 10 min. The solubilized gel was loaded onto binding columns and centrifuged at 12,000×g for 1 min. The flow-through was discarded and the column was washed once with buffer PE containing ethanol. The DNA bound to the column was eluted using the elution buffer provided with the kit, or alternatively with nuclease-free water. The concentration of the obtained DNA was estimated by measuring the absorbance at 260 nm (A_{260}) and using the following formula: DNA concentration = A_{260} × 50 × dilution factor.

3.2.2.6. Sub-cloning of PCR products into pGEM-T<sub>Easy</sub> vector

The DNA fragments eluted from the agarose gel were cloned into pGEM-T<sub>Easy</sub> vector which allows efficient sequencing using the common sequencing primers T7 and SP6. 3 μl of eluted DNA (1 μg/μl) was ligated with 1 μl of pGEM-T<sub>Easy</sub> vector (50ng) in the presence of 1 μl of T4 DNA ligase in a 10 μl reaction volume. The reaction was allowed to proceed at 4°C for 16 h following which 8 μl of the ligation mix was used to transform DH5α strain of E.coli following standard protocols. The transformation mix was spread onto LB-agar plates containing appropriate ampicillin (100 μg/mL) and the blue-white selection reagent (40 μl/plate). The plate was incubated at 37°C for 12 h following which the white colonies were picked up for screening for presence of the gene of interest.

3.2.2.7. Preparation of ultra competent DH5α cells

Ultra competent cells were prepared by using Inoue method (Inoue et al., 1990). Briefly, the DH5α cells were grown in the SOB culture medium (20 g/l Tryptone, 5 g/l yeast extract, 0.5
gm/l sodium chloride, 2.5 mM potassium chloride and 10 mM magnesium chloride, pH 7.0) at 18°C till the O.D.₆₀₀ of 0.55 was attained, the flaws were then shifted to ice-water bath for 10 min. The cells were harvested by centrifugation at 2500×g, all media was discarded and the cell pellet was resuspended in Inoue transformation buffer (55 mM manganese chloride, 15 mM calcium chloride, 250 mM potassium chloride, 10 mM PIPES, pH 6.7). The suspension was centrifuged at 2500×g, the buffer was discarded and cell pellet was resuspended in fresh Inoue transformation buffer, DMSO (1.5 ml/20 ml of buffer) was added and the cells were frozen at -70°C. Cells were checked for transformation efficiency and were used if transfection efficiency was above 5×10⁸ transformed colonies/µg of DNA.

3.2.2.8. Miniprep plasmid DNA isolation

The plasmid isolation was done using Qiagen kit (Hilden, Germany) using the alkaline lysis protocol. The culture, grown overnight, was harvested by centrifugation and then resuspended in P1 buffer (50 mM Tris-HCI, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0). The cells were then lysed by addition of P2 buffer (200 mM sodium hydroxide, 1% SDS) and incubated at room temperature for 3-5 min. The plasmid DNA was then renatured using P3 buffer (3M potassium acetate, pH 5.5) while the genomic DNA and proteins were precipitated. The plasmid DNA obtained in supernatant after centrifugation at 13000×g for 10 min was precipitated by addition of 0.7 volume of isopropanol. The plasmid was then washed with 70% cold ethanol and the pellet air dried. The pellet was then dissolved in sterile water or 10 mM Tris-HCl, pH 8.0 for further use.

3.2.2.9. DNA sequencing

DNA was sequenced by the di-deoxy method (Sanger et al., 1977) at the DNA sequencing facility, Department of Biochemistry, University of Delhi, South Campus, New Delhi, India.

3.2.2.10 Generation of pXG-GFP-Prohibitin and pXG-GFP-Prohibitin N252D vectors

The prohibitin gene fragment was released from pGEMT-Prohibitin clone 3 (2 µg) (confirmed by DNA sequence analysis to have the complete gene) using Not I and Nde I (to cut the pGEMT backbone) for restriction digestion also in a separate reaction pXG-GFP+2' (2 µg) was digested using Not I. The digested pXG-GFP+2' vector was dephosphorylated at the overhangs using Shrimp alkaline phosphatase, Roche (Basel, Switzerland) as per the manufacturer's protocol; briefly, 2 µg of digested DNA was dephosphorylated using 1U of the enzyme for 20 min at 37°C. After which both the phosphatase treated pXG-GFP+2' vector and the digested pGEMT-Prohibitin vector were heated to 65°C for 20 min to heat inactivate the enzymes. The SAP treated vector pXG-GFP+2' vector and the released prohibitin fragment were then ligated at the ratio of approximately 1:3 (Vector: Insert) using Quick ligation kit from New England Biolabs (Beverly, MA).
strictly following the manufacturer's protocol. 4\mu l of the ligation mixture was used to transform the DH5\alpha cells, the obtained colonies were screened using colony PCR for prohibitin, the positive clones were used for plasmid purification using Qiagen Miniprep Kit (Hilden, Germany) following manufacturers protocol, confirmed by restriction digestion using Not I for release of prohibitin fragment and Bgl II for checking the orientation of the clone. The clones with the correct orientation were then sequenced for confirming the complete sequence and ruling out any mutations in the clones before transfection. The sequencing spanning the whole fusion product was done using the primer pairs listed below:

<table>
<thead>
<tr>
<th>Region</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>5'- CGCCTCTCTCTCTCTCTC - 3'</td>
<td>5'- GTCCTCTTGAAGTCGAT -3'</td>
</tr>
<tr>
<td>GFP-Pro 1</td>
<td>5'- AGCAGCAGCAGACTTCTTC -3'</td>
<td>5'- GCAACTTTCGACATAATCG -3'</td>
</tr>
<tr>
<td>GFP-Pro 2</td>
<td>5'- CACTACCAGCAGAACCACC -3'</td>
<td>5'- ATTTTCGTTGCTGACAGAG -3'</td>
</tr>
<tr>
<td>Pro 1</td>
<td>5'- GTACAGCTGCTGCTTTGT -3'</td>
<td>5'- CTGCTCATTCTCCATCAC -3'</td>
</tr>
<tr>
<td>Pro 2</td>
<td>5'- CAGTTCGGTCTCTCATC -3'</td>
<td>5'- GAGAAGCAAGGAGAGAGG -3'</td>
</tr>
</tbody>
</table>

GPI site mutant was generated using overlap extension using polymerase chain reaction using Quick change Site directed mutagenesis kit from Stratagene strictly following the manufacturer's protocol where asparagine 252 was replaced by aspartic acid using the following primers;

1. N252D Prohibitin sense, 5'-CCAGATCGTGCCCCATGCAGGACGTGACGTTCGTACCGAAAG-3'
2. N252D Prohibitin antisense, 5'-CTTTCGGTACGAACGTCACGTCCTGCATGGCACGATGTGG-3'

The clones were confirmed for the desired mutations using DNA sequencing.

3.2.2.11 Transfection of *L. donovani* cells

Transfection of *L. donovani* cells with pXG-GFP+2, pXG-GFP-Prohibitin and pXG-GFP-Prohibitin N252D was performed as described earlier (Robinson and Beverley, 2003). Cells from stationary phase grown in liquid culture were harvested at 805xg for 10 min. The cells were then washed with twice with Cytomix buffer (120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4, 25 mM HEPES, 2 mM MgCl2, 2 mM EDTA, pH 7.6) with at least half the volume of the initial culture medium. The cells were counted after the washing and the cell density adjusted to 1x10^8 cells/ml. To the 4mm electroporation cuvette, BTX (San Diego, CA) 25 \mu g of the vector DNA added in not more than 50 \mu l of buffer followed by addition of 500 \mu l of the cell suspension, electroporate twice at 1500V, 25\mu F and \approx \Omega with 10 sec gap between the two pulses. The cells were kept on ice and
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then transferred to 10 ml of M199 supplemented with 10% fetal bovine serum overnight at 22°C. The next day, antibiotic selection was started by supplementation of G418 at 10μg/ml; the concentration of G418 was doubled every few days depending on the growth of the culture till 1 mg/ml concentration was achieved. Alternatively, cells were plated on agar plates having G418 and individual colonies were picked and screened. Cells were routinely screened for GFP positivity and the expressions of the overexpressed proteins were confirmed by RT-PCR, immunofluorescence and Western blotting.

3.2.2.12 SDS-PAGE and Western blotting

For preparing whole cell lysates, cells were lysed in 2xSDS-gel electrophoresis sample buffer (0.125 Tris-HCl, pH 6.8; 4% SDS; 20% glycerol and 10% 2-mercaptoethanol). The protein was estimated using CB-X™ protein assay kit using a modified Bradford protocol (Bradford, 1976). 1 ml of prechilled precipitation reagent was added to 10 μl of the sample and centrifuged at 12,000×g for 10 min at 4°C, the supernatant was discarded and the pellet were allowed to air dry after which 50 μl of solublization buffer I and II were added. Samples were vortexed vigorously and then 1 ml of coloring reagent was added and 200 μl of the sample read spectrophotometrically at 595nm. The protein concentrations were determined from the standard curve produced using Bovine serum albumin as a standard. Samples were analyzed on 12% reducing SDS-PAGE. The gels were run in Tris-Glycine buffer containing 25mM Tris base, 192mM Glycine and 0.1% SDS w/v at 60V constant, broad range molecular weight marker (Amersham, NJ) was used for molecular weight determination of the protein bands. The proteins were transferred onto nitrocellulose membranes using a BioRad Western transfer apparatus. The blots were incubated with 5% blotto (Non-fat dry skimmed milk) in 0.05% PBS-Tween 20 for 1 h to block non-specific binding sites following which they were incubated for 1 h with primary antibody at an appropriate dilution prepared in 1% blotto in 0.05% PBS-Tween-20. The blots were washed thrice with 0.05% PBS-Tween-20 at 5 min intervals following which they were incubated for 1 h with secondary antibody at an appropriate dilution. The primary antibodies for prohibitin, HSP-70, phospho-tyrosine, phosphothreonine, Ubiquitin, GFP, β-actin and α-tubulin were used at dilutions of 1:1000 and secondary antibodies used at 1:5000 dilution, cTXNPx antibody was used at 1:5000 dilution with the secondary antibody being used at 1:10000. For visualization, ECL plus detection reagent from Amersham (Uppsala, Sweden) was used as per the manufacturer's guidelines the resultant exposure was recorded on X-ray films from Kodak (India). It is important to note that the blots for probing phosphorylated proteins were performed using 1% BSA as blocking agent instead of blotto.
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3.2.2.13 Immunoprecipitation of prohibitin

For immuno-precipitation, promastigote from stationary phase cultures were taken and washed 3X with phosphate buffered saline, pH 7.4 at 4°C. The cells were then lysed on ice for 10 min using M2 lysis buffer (50 mM Tris HCl, 10% glycerol, 1% Triton X-100, 0.5 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, pH 7.4), the cell suspension was then centrifuged at 3200×g for 10 min at 4°C. The supernatant was collected and the concentration of protein was estimated. Meanwhile, protein G beads were washed with M2 lysis buffer and the washing repeated three times, antibody was added to the beads at a concentration of 2 μg/30 μl of beads for 6 hrs at 4°C after which the unbound antibody was removed keeping only the antibody bound beads for immunoprecipitation. Approximately; 2 mg of protein was used for each immunoprecipitation containing 2 μg of antibody bound to protein G beads and the reaction was incubated for 8-12 hrs on an end to end shaker at 4°C. After the reaction, the beads were washed extensively using M2 lysis buffer to remove any non specific interaction, lysed in 2×SDS sample buffer, resolved on 12% SDS-PAGE, Western blotted or silver stained for sequencing.

3.2.2.14 Silver staining and protein sequencing

For silver staining; Vorum's protocol (Mortz et al., 2001) was used. Briefly, gels were fixed with fixing buffer (50% methanol, 12% acetic acid, 0.05% formalin) overnight, washed three times with 35% ethanol for 20 min each followed by twice with water. The gels were then sensitized by treating for 2 min with sensitizer solution (100 mM sodium thiosulphate and 30 mM potassium ferricyanide) followed by four times washing with water for 20 min each. The gels were then stained for 20 min with 0.2% Silver nitrate solution containing 0.076% formalin. The gel was washed once with water for 1 min before being developed with developing solution (6% sodium bicarbonate, 0.05% formalin and 0.0004% sodium thiosulphate) till the bands appeared to the desired intensity after which the reaction was stopped using 50% methanol and 12% acetic acid. Stained gels were kept in 1% acetic acid and were washed with water before cutting the band for sequencing.

For sequencing, protein bands were excised from silver stained gels and given for protein sequencing of tryptic digests of the excised proteins at the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia Biomedical Research Facility, University of Virginia, VA where the Sequest search algorithm (Bioworks software, Thermoelectron Corporation, Waltham, MA) was used to determine cross-correlation scores between acquired spectra and the Leishmania major database (GeneDB, Sanger Institute, UK). The protocol described briefly; the gel was transferred to a siliconized tube and washed and destained in 200 μl 50% methanol overnight. The gel pieces were dehydrated in acetonitrile, rehydrated in 30 μl of 10 mM dithiothreitol in 0.1 M
ammonium bicarbonate and reduced at room temperature for 0.5 h. The DTT solution was removed and the sample alkylated in 30 μl 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The reagent was removed and the gel pieces dehydrated in 100 μl acetonitrile. The acetonitrile was removed and the gel pieces rehydrated in 100 μl 0.1 M ammonium bicarbonate. The pieces were dehydrated in 100 μl acetonitrile, the acetonitrile removed and the pieces completely dried by vacuum centrifugation. The gel pieces were rehydrated in 20 ng/μl trypsin in 50 mM ammonium bicarbonate on ice for 10 min. Any excess trypsin solution was removed and 20 μl 50 mM ammonium bicarbonate added. The sample was digested overnight at 37°C and the peptides formed extracted from the polyacrylamide in two 30 μl aliquots of 50% acetonitrile/5% formic acid. These extracts were combined and evaporated to 25 μl for MS analysis.

The LC-MS system consisted of a Finnigan LTQ ion trap mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm x 75 um id Phenomenex Jupiter 10 um C18 reversed-phase capillary column. 0.5-5 μl volumes of the extract were injected and the peptides eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 μl/min. The nanospray ion source was operated at 2.8 kV. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 1000 CAD spectra of ions ranging in abundance over several orders of magnitude.

3.2.2.15 Recombinant prohibitin expression

Prohibitin sequence was cloned from pGEMT-Prohibitin into pET 28a+ using Not I restriction enzyme and strategies described under molecular biology techniques (3.2.2). The sequence confirmed plasmid was then transformed into BL21 cells and screened for expression. The protein expression was found to be highest at 18°C using 0.1 mM IPTG for induction. The protein was found to be exclusively present as inclusion bodies hence denaturing conditions were used for purification of the protein as per Qiagen protocol and recommendations. Briefly, 1 gm of the cell pellet obtained after induction was lysed in 50 ml of buffer B (100mM Sodium dihydrogen phosphate, 10 mM Tris chloride and 8M Urea, pH 8.0), after vortexing for 30 min to ensure complete lysis, the supernatant was centrifuged at 13000×g for 10 min to pellet cellular debris. The supernatant was then loaded onto Ni-NTA column packed volume of 0.5 ml and allowed to flow through at not more than 0.5 ml/min for efficient binding. The column was washed with 250 ml of
buffer C (100mM Sodium dihydrogen phosphate, 10 mM Tris chloride and 8M Urea, pH 6.3) to remove unbound proteins. The protein was eluted with 10 ml of buffer E (100mM Sodium dihydrogen phosphate, 10 mM Tris chloride and 8M Urea, pH 4.5). The eluate was then concentrated on Amicon 10 concentrator and several serial dilutions with PBS were performed to remove urea and refold proteins at 4°C. The final sample was concentrated to 250 µl, aliquoted and stored. The purity of purification was tested using SDS-PAGE followed by commassie staining and Western blotting.

3.2.2.16 Transcription translation coupled rabbit reticulocyte system

Recombinant prohibitin was generated using TNT® coupled reticulocyte lysate systems Promega Corporation (Madison, WI). Prohibitin gene encoding T7 promoter based plasmid (1 µg) was added to the reticulocyte lysate reaction (TNT lysate, 40U RNasin, 2 mM complete amino acid mixture and 1µl T7 RNA polymerase) at 37°C for 1 h; a parallel reaction was carried out without the presence of prohibitin encoding plasmid as a control. The macrophage membrane fractions (50~g/reaction) were added and the mixture was immunoprecipitated with anti-prohibitin antibody as described previously, resolved on 12% SDS PAGE, silver stained, processed for tryptic digestion and microsequencing.

3.2.3 Cell Biology and other techniques

3.2.3.1 Immunocytochemistry
For visualization of mitochondrion and prohibitin staining, cells were incubated with 0.5 µM Mitotracker® Red for 10 min and subsequently fixed with 2% formaldehyde for 20 min and washed 3X with phosphate buffered saline twice for 5 min each. To block non-specific binding sites, cells were incubated with 3% normal goat serum containing 0.1% saponin for 30 min and subsequently incubated with anti-prohibitin antibody (1:50) in the presence of saponin for 1 h at 37°C, the cells were then washed thrice with phosphate buffered saline. Staining with Alexa-fluor 488 conjugated secondary antibody (1:100) for 1hr at 37°C was used to visualize prohibitin staining, the nucleus and kinetoplast were stained with 1 µg/ml Hoechst 33342 for 10 min after which the cells were washed and mounted on a slide. Cells were observed under Nikon Eclipse TE 2000 E confocal microscope using Argon laser (488 nm) and HeNe laser (513 nm) and overlapped images were generated. Data for co-localization was obtained by Image-Pro Plus™ version 5.0 software (Media Cybernetics, Silver Spring, M.D.).
3.2.3.2 Live cell staining for surface prohibitin

For visualization of prohibitin staining on live cells, the above mentioned protocol was used without fixation and saponin permeabilisation. Briefly, live cells were suspended in phosphate buffered saline containing glucose and subsequently incubated with anti-prohibitin antibody (1:25) for 1 h at 4°C. After three washes with the phosphate buffered saline, cells were incubated with Alexa- fluor 488 conjugated secondary antibody (1:100) for 1 hr at 4°C, the cells were then visualized under a Nikon Eclipse TE 2000 E confocal microscope. For flagellar pocket labeling, FM-464 (10μM/10^6 cells at 4°C for 10 min) was used to stain the flagellar pocket membranes of live cells stained for prohibitin. Flow cytometric analyses of 50,000 cells per group (Live cells for surface staining and fixed cells for intracellular staining, the antibody concentration being same for both groups) were performed on a BD-LSR flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed using Win-MDI shareware as described previously (Iyer et al., 2008). For staining of surface and intracellular prohibitin; live cells were stained with rabbit anti-human prohibitin (1:50) antibody followed by Alexa Fluor 488 conjugated secondary antibody (1:100) as described above for surface staining. The cells were then fixed and permeabilized for staining with murine anti-human Prohibitin Antibody (1:50) followed by Alexa Fluor 594 secondary antibody (1:100) for intracellular labeling as described previously. Live cell staining for surface HSP70 was performed by incubating live macrophages with anti-HSP70 antibody (1:50) and Alexa Fluor 488 conjugated secondary antibody (1:100) the rest of the protocol being similar to live cell staining described above.

3.2.3.3 Preparation and staining of L. donovani 'ghost' membranes

Membrane preparation was performed using the protocol described earlier (Snapp and Landfear, 1997). Briefly, 2×10^7 cells were washed in ice-cold PBS. The pellet of cells was resuspended in MME (10 mM MOPS, pH 6.9, 0.1 mM EGTA, 1 mM MgSO4, and 0.1% Triton X-100) containing protease inhibitors. The cells were incubated on ice for 10 min, and were centrifuged at 3,000xg. The pellet was washed once with PBS and lysed in sample buffer. The supernatant was concentrated to 1/10 of the original volume with a Microcon 10 microconcentrator, Millipore (Billerica, MA) treated with sample buffer, equal protein was loaded in SDS-PAGE gel, resolved and Western blotted. For immunofluorescence, the pellets were prepared as described above, except that they were resuspended in PBS after centrifugation and washing. Isolated membranes were fixed using the protocol described above and stained using α-tubulin (1:100) followed by the Alexa Fluor 594 conjugated secondary antibody and then the prohibitin staining was performed. The cells were scanned using a Nikon Eclipse TE 2000 E confocal microscope as described above.
3.2.3.4 Drug treatments
Paclitaxel at doses of 0, 10, 20, 30 μM were added to Leishmania donovani promastigote culture in M199 medium for 24 h after which they were processed for various investigations. For HSP inhibition experiments, cells were incubated with 250 μM of HSP Inhibitor 1, 30 min prior to addition of 30 μM paclitaxel. For autophagy experiments, inhibitors of autophagy like 10 mM 3 - Methyl adenine and 200 nM Bafilomycin A1 were added 30 min prior to the addition of 30 μM paclitaxel.

For antisense experiments, the cells were preincubated for 30 min with 40 μM anti-prohibitin antisense, Qiagen (Hilden, Germany) (5'-C*T*T*C*T*G*C*A *G*C*A*A*C*T*T*C*G*A* C*A*T-3') or scrambled antisense (5'-C*A*T*C*G*A*C*T*T*C*A*A*C*G*C*T*G*C*T*T-3') oligo-nucleotides having phosphorothioation at all bonds.

3.2.3.5 Assay for detection of apoptosis by Annexin-V/PI staining
The Vybrant apoptosis assay kit was used to perform Annexin-V/PI staining as described previously (Iyer et al., 2008). The cells after appropriate treatment were harvested by centrifugation at 805×g for 10 min and were given two washes with ice-cold phosphate buffered saline following which they were resuspended in 100 μl of ice-cold 1X Annexin binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl₂, pH 7.4). 5 μl of Annexin-V conjugated to Alexa fluor 488 and 1 μl of working solution of PI (100 μg/ml) were added to the 100 μl cell suspension. Cells were incubated for 15 min at room temperature. Following this, 400 μl of 1X Annexin binding buffer was added to dilute the sample. The samples were placed on ice. The fluorescence was measured by flow cytometry in FL1 and FL2 channels for Annexin-V-Alexa fluor 488 and PI fluorescence respectively.

3.2.3.6 Monodansyl cadaverine staining and measurement for autophagy
Autophagic vacuoles and lysosomes in L. donovani were labeled with 50 mM MDC and 1 μM LysoTracker® red respectively in Medium 199 supplemented with 10% fetal bovine serum at 22°C for 10 min as described earlier (Munafo and Colombo, 2001) after which the cells were washed thoroughly with ice cold phosphate buffered saline and observed under Nikon Eclipse TE 2000 E confocal microscope as described above. Intracellular MDC labeling was measured fluorometrically (excitation wavelength 380 nm, emission wavelength 525 nm) by lysing the stained cells in lysis buffer (10 mM Tris and 0.1% Triton X-100, pH 8.0), the readings were normalized to cell numbers.
3.2.3.7 Transmission electron microscopy

For electron microscopy, cells were fixed in Karnovsky's fixative (4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer) for 30 min at room temperature and processed for resin block preparation using standard protocols. Thin sections were transferred to grids and examined with a Morgagni 268(D) Transmission Electron Microscope, FEI Company (Hillsboro, OR) using the Electron Microscopy facility of the All India Institute of Medical Sciences, New Delhi, India.

3.2.3.8 Procyclic and metacyclic separation

Procyclic and metacyclic promastigotes were separated by incubating cells from stationary phase culture with 100 µg/ml of PNA for 1 h in phosphate buffered saline. The agglutinated procyclic cells were separated from free metacyclics by differential centrifugation, using 100xg to pellet the agglutinated procyclic and 1000xg to pellet metacyclics using an eppendorf centrifuge (Model 5810R, Eppendorf AG, Hamburg, Germany).

3.2.3.9 PI-PLC treatment

2x10⁶ control or heat shocked cells were taken and washed 2X with buffer containing 10mM Tris and 144mM NaCl, pH 7.4. The cells were suspended in the buffer and treated with 0.5 units of PI-PLC at 37°C for 1 hr. The suspension was pelleted at 1000xg and the supernatant was biotinylated using 1 mg of NHS-Sulfo linked biotin, Pierce (Piscataway, NJ). The excess biotin was removed using Amicon 10 concentrator, Millipore (Billerica, MA) with several washings with PBS and the resultant concentrate was used to immunoprecipitate prohibitin as described previously, resolved on 12% SDS PAGE and Western blotted using Streptavidin antibody. For Intracellular prohibitin levels, cells after treatment were lysed in 2xSDS Lysis buffer. Cells treated with PI-PLC were also taken and used for live cell staining followed by flow cytometry as described previously. For the recovery experiments, PI-PLC treated cells after treatment were returned to 22°C in M199 supplemented with 10% fetal bovine serum, aliquots of cells were taken at various time points and live cell prohibitin staining as described previously was done to observe the repopulation of prohibitin onto the surface.

3.2.3.10 Generation of Fab fragments of anti-prohibitin antibody

For neutralization of surface prohibitin of Leishmania donovani, cells were treated with Fab fragments of anti-prohibitin antibody, prepared by digesting 120 µg of the antibody with 6 µg of papain at 37°C for 1 hr, the reaction was stopped using 90 mM iodoacetamide the excess of which was removed by serial dilutions and concentration using Amicon 10 concentrator, Millipore (Billerica, MA). Undigested antibody was removed from the supernatant with Protein G beads and
the Fab fragments were incubated with live PKH labelled parasites at 4°C for 1 h after which they were used for infecting macrophages. Fab fragments of anti-cytosolic tryparedoxin antibody were used to incubate control parasites. Binding efficiency of the parasites was determined by flow cytometry and microscopy as described above in section 3.2.1.6.

3.2.3.11 Prohibitin coating on coumarin encapsulated beads and binding assay
Coumarin encapsulated beads were obtained from the laboratory of Dr Amulya Panda, NII, India, prepared as described previously (Kanchan and Panda, 2007). Equal numbers of beads were coated at 37°C for 1 hr using 20 μg of prohibitin or bovine serum albumin. Beads were added to macrophage culture at MOI (multiplicity of infection) of 1:1 and incubated for 1 hr after which the unbound beads were washed and the cells flushed for flow cytometric analysis or microscopic analysis.

3.2.3.12 Macrophage membrane preparation
Sub-cellular fractionation of J774A.1 macrophages was performed after lysis with hypotonic buffer. J774A.1 macrophages after appropriate treatment were allowed to swell for 10 min in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.5) followed by homogenization with 50 strokes using a Dounce homogenizer. More than 90% cellular lysis was ensured by visualizing under a light microscope, and immediately after lysis, the mitochondrial membranes were stabilized by addition of 2.5X mitochondrial stabilization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, 2.5 mM EDTA, pH 7.5) to a final concentration of 1X. The homogenate was centrifuged at 1300×g for 15 min to isolate the nuclear fraction. The post-nuclear fraction was further centrifuged at 17,000×g for 15 min in an ultracentrifuge (Beckman Optima XL-100K ultracentrifuge) to isolate the mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000×g for 1 h to obtain the membranous fraction as a pellet and the supernatant obtained was the cytosol. The homogeneity of the obtained fractions was determined by probing for fraction specific proteins by Western blotting.

3.2.3.13 Enzyme linked immunosorbent assay
Sera from active VL patients were obtained with the consent of donors, patients and in case of minors from their parents/guardians as approved by the Institutional Human Ethical Committee of the Indian Institute of Chemical Biology, Kolkata. Diagnosis of VL was based on microscopic demonstration of Leishmania spp. amastigotes in splenic aspirates according to WHO recommendations. Crude parasite leishmanial antigen (1 μg/well) or peptide A (RRAAVVR, provisional patent application filed, 474/DEL/2009) (2 μg/well) were coated in 96-well microtitre plates and incubated overnight at 4°C with sera (1:250) from active VL patients/normal controls that
were layered on the antigen. Following wash, binding was colorimetrically detected using protein-A-HRP (1:2000) using azinobisthiosulphuric acid (ABTS) as the substrate (Chatterjee et al., 1999). Each point represents the average of four independent experiments.

3.2.3.14 Bioinformatics
The prohibitin sequence was aligned with prohibitin sequences from other phyla using ClustalW hosted at the European Bioinformatics Institute (Chenna et al., 2003) using default parameters including Gonnet scoring matrix, a gap penalty of 10 and a gap extension penalty of 0.2. Softwares like Prosite were used to identify signature motifs and Target P (CBS, Technical University of Denmark) (Emanuelsson et al., 2000) and Phobius (EMBL-EBI) (Kall et al., 2004) for prediction of signal sequence. Big PI software (Eisenhaber et al., 1999; Eisenhaber et al., 2000) was used to predict the possibility of GPI anchors.

3.2.3.15 Densitometry and statistics
Densitometric measurements for quantitation of signals on immunoblots or ethidium bromide stained agarose gels were performed using a UVP Gel Documentation instrument, and the acquired data was analyzed on LabWorks image analysis and acquisition software (UVP, v.4.0.0.8). Data from at least 3 experiments were quantitated to arrive at the average value of the signal. All measurements were normalized to internal loading controls. To determine statistical significance, the data was analyzed by Student’s t-test and the values were expressed as mean±SEM. The values were considered to be significantly different at p<0.05.

3.2.3.16 Flow cytometric analysis
Flow cytometric analyses of 50,000 cells per group for the stained L. donovani cells and 20,000 cells per group for macrophages were performed on a BD-LSR flow cytometer, Becton Dickinson (San Jose, CA) and data analyzed using Win-MDI shareware as described previously (Iyer et al., 2008).
3.3 Instrumentation

Optiphot fluorescence microscope, E600W fluorescence microscope and T2000E Confocal microscope C1 were from Nikon (Tokyo, Japan).

Multitemp III water bath and EPS 500/400 power supply were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Gyratory water bath shaker was purchased from New Brunswick Scientific Co., Inc (Edison, NJ).

Centrivac and Biofuge table top centrifuge were from Heraeus (Allerod, Denmark).

μ-Quant microplate reader was from Bio-tek Instruments Inc. (Winooski, VT).

Gene Pulser X cell, Protean II polyacrylamide gel system and Mini Trans blot system were from Bio-Rad Laboratories (Hercules, CA).

Submarine DNA electrophoresis system was procured fro Bangalore Genei (Bangalore, India).

Laminar flow hoods were purchased from Kartos Ltd. (New Delhi, India).

Eppendorf 5810R centrifuge was purchased from Eppendorf (Hamburg, Germany).

LS50B flourimeter was from Perkin Elmer Biosystems (Norwalk, CT).

BD-LSR was from Bectinson Dickinson Biosciences (San Jose, CA).

Peltier Thermal Cycler-200 was purchased from MJ research (Waltham, MA).

Doc-It Gel Documentation system was procured from UVP Bio Imaging System Incorporation (Upland, CA).

MegaTech BOD incubator was obtained from MegaTech International (Mumbai, India). CO₂ incubators were from Thermo Electron Corporation (Marietta, Ohio).
Bibliography


