Chapter 3

Materials, Methods and Instrumentation
3.A. Materials

Dulbecco's Modified Eagle's medium (DMEM), Rosewell Park Memorial Institute medium (RPMI – 1640), and gentamycin were purchased from GIBCO BRL (Gaithersburg, MD). Fetal calf serum (FCS) and DMEM without phenol red were procured from Biological Industries (Kibbutz Beit Haemek, Israel). Medium 199 (M199), Geniticin sulphate (G418) and Ampicillin were purchased from Sigma Chemical Company (St. Louis, MO). Brain Heart Infusion Agar was procured from Pronadisa (CONDA Laboratories, Madrid, Spain). LB Agar and LB Broth Base were obtained from Invitrogen Life Technologies Corporation (Carlsbad, CA). Cellulose acetate and cellulose nitrate membrane filters were obtained from Sartorius (Sartorius AG, Goettingen, Germany).

Deoxy-ribonucleotide (dTNP) mix, MgCl₂, and pGEM-T Easy sequencing vector were procured from Promega (Madison, WI). Taq DNA Polymerase, BglIII, BamHI, EcoRI, HindIII and NotI were from New England Biolabs (Beverly, MA). Platinum Gold High Fidelity Taq Polymerase (HiFiTaq), Superscript II First Strand Synthesis kit and TriZOL reagent were procured from Invitrogen Corporation (Carlsbad, CA). 100bp and 1kb DNA ladder were obtained from MBI Fermentas (Ontario, Canada). 6X DNA loading dye was obtained from GIBCO BRL (Gaithersburg, MD). Synthetic oligo-nucleotides were obtained from Sigma GENOSYS (Bangalore, India), Microsynth (Germany) or Biobasic, Inc (Ontario, Canada). Plasmid MiniPrep kit and EndoFree Plasmid Maxi kit were obtained from Qiagen (GmbH, Hilden). GenElute™ Mammalian Genomic DNA Miniprep kit was obtained from Sigma Chemical Company (St. Louis, MO).

CB-X Protein Assay kit was purchased from G-Biosciences (St. Louis, MO). Ammonium persulphate (APS) and N, N, N', N' - tetramethylethylene-diamine (TEMED) were obtained from Sigma Chemical Company (St. Louis, MO). Rainbow™ protein molecular weight markers were obtained from Amersham Pharmacia Biotech. (Uppsala, Sweden). Mouse monoclonal anti-GFP and mouse polyclonal anti-chSC73/iHSP72 antibodies were purchased from Stressgen (Victoria, BC Canada). Rabbit polyclonal anti-HSP90 antibody was obtained from Santa Cruz Biotechnology Inc. (California). Heat Shock Protein Inhibitors I and II were obtained from Calbiochem (Darmstadt, Germany). Horse Radish Peroxidase (HRP) - conjugated anti-rabbit IgG and anti-mouse IgG or anti-mouse IgM antibodies were purchased from Jackson Research (West Grove, PA). All Alexa Fluor
labelled secondary antibodies were from Molecular Probes (Eugene, OR). Vector VIP Peroxidase Substrate kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). Enhanced Chemi-luminescence (ECL) components (Hyper Cassette Blue, Hyper-film ECL) were obtained from Amersham Bioscience (Amersham, Piscataway, NJ). ECL reagents A and B were obtained from the EZ-ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel).

Chloroform, Isopropyl-alcohol, Di-sodium hydrogen phosphate, Sodium dihydrogen phosphate, Sodium chloride, Glycerol, Glycine, Acetic acid, Hydrochloric acid, Sulfuric acid, Tris, Tris-HCl, Potassium chloride, Di-potassium hydrogen phosphate, Formaldehyde, Phenol, H₂O₂ and Methanol (HPLC grade) were obtained from E. Merck (Bombay, Maharashtra, India). Ethanol was obtained from Fluka Chemie GmbH (Buchs, Switzerland).

Hemin, Sodium bicarbonate, Sodium lactate, L-glutamine, Propidium iodide, Coomassie Blue R-250, Bromophenol blue, Acrylamide, N, N’- methylene – bis -acrylamide, Tween-80, Agarose, EDTA, EGTA, SDS, HEPES, L-ascorbic acid, Triton X-100, Magnesium chloride, Calcium chloride, Glucose, Blue White Select Screening Reagent, Proteinase K, RNase A, Ethidium bromide, Calcium Ionophore, Cyclosporin A, Diethyl pyrocarbonate (DEPC), Dimethyl Sulfoxide (DMSO), Monodansyl Cadaverine, Ionomycin, Potassium Antimony Tartarate and UltraPure N-acetyl L-cysteine were obtained from Sigma Chemical Company (St. Louis, MO).

Vibrant™ Apoptosis Assay kit #2, Hoechst, JC-1 (5,5', 6,6'-tetrachloro- 1,1', 3,3'-tetraethylbenzimidazolyl carbocyanine iodide), Fluo-3AM (fluo-3 acetoxy-methyl ester), Rhod-2AM, CM-H2DCFDA (5-(and-6) chloromethyl-2’, 7’-dichlorodihydrofluorescein diacetate, acetyl ester), Syto Green 11 and Pluronic F127 were obtained from Molecular Probes (Eugene, OR). DeadEnd™ TUNEL Apoptosis Detection System was purchased from Promega (Madison, WI).

All other chemicals, unless specified, were of the highest reagent grade and were purchased from Sigma Chemical Company (St. Louis, MO). All plastic-ware was purchased from Nunc (Denmark), BD Falcon (Franklin Lakes, NJ), Axygen (Union City, CA) or Griener Bio-one (Frickenhausen, Germany).
3.B. Methods

3.B.1. Cell Culture Techniques

3.B.1.1. Macrophage Strains and Cultures

The murine macrophage cell line J774A.1 (ATCC no. TIB-67) was maintained in DMEM or RPMI without phenol red adjusted to a final concentration of 4mM L-glutamine and supplemented with 10% (v/v) heat inactivated (45 minutes at 65°C) fetal calf serum, at 37°C in an atmosphere of 5% CO₂ and air.

Macrophages were sub-cultured every 72 hours, or when, approximately 70% confluent. The supernatant, with dying non-adherent macrophages, was aspirated out and discarded. The adherent monolayer of healthy macrophages was flushed out by repeated pipetting with excess of plain media and harvested by centrifugation at 290 x g for 5 minutes. The pellet was washed once with plain media, and cell counts estimated using a haemocytometer. Based on these counts, macrophages were resuspended and plated in an appropriate volume of complete media. These were incubated under conditions mentioned above. Freshly plated cells were rounded in appearance. Within a few hours (ideally 6 - 8 hours) at 37°C, healthy macrophages became adherent, assumed amoeboid morphologies, and displayed little granularity.

3.B.1.2. Parasite Strains and Cultures

Promastigote cultures of a cloned cell line of Leishmania donovani (MHOM/IN/80/DD8) were obtained from Dr. R. Vishwakarma from National Institute of Immunology, New Delhi, India. These were maintained on slants or in liquid cultures; or used for the generation of axenic amastigotes.

3.B.1.2.a. Slant Cultures of Promastigotes

Routine cultures of promastigotes were maintained on solid blood agar slants as described previously (Sengupta et al., 1999; Das et al., 2001).Briefly, these slants
contained 1% glucose, 5.2% brain heart infusion agar extract, and 6% (v/v) rabbit blood with gentamycin at a final concentration of 1 – 1.5 mg/mL of medium at 26°C.

Inoculum for slant cultures was derived from large-scale liquid cultures (approximately 10⁹ cells) of promastigotes. Dead cells were pelleted down by centrifugation at 129g for 10 minutes at 26°C. The supernatant was carefully decanted into a fresh polypropylene tube, live cells harvested by centrifugation at 2000 x g for 8 minutes and washed thrice with excess of plain modified DMEM to remove all traces of FCS. The pellet obtained after the final wash was resuspended in residual media, transferred to the slant surface and spread evenly with a flame sterilised bacterial loop. Growth was usually detected after 4 – 7 days at 26°C.

Slants were sub-cultured when confluent: usually after 48 – 72 hours of growth. A loop-full of inoculum was transferred from the confluent slant to a fresh slant (thawed at 26°C for an hour before sub-culturing) and spread out evenly on its surface before incubation at 26°C to allow growth.

3.B.1.2.b. Liquid Cultures of Promastigotes

Routine cultures of promastigotes were maintained at 26°C in modified DMEM (1 litre of DMEM was supplemented with 5.96g of HEPES, 5mg of hemin, 0.5mL of triethanolamine, 40mg of Tween-80, 13.36mg of adenine, 7.60mg of xanthine, 1mg of biopterin, and 3.7g of sodium bicarbonate and adjusted to a pH of 7.2) containing gentamycin at a final concentration of 1 – 1.5 mg/mL and 10% (v/v) FCS.

Inoculum for initiating liquid cultures was derived from a healthy 48 - 72 hour slant culture. Parasites on the slant were flushed out by repeated pipetting with plain modified DMEM, thawed to room temperature. A uniform cell suspension was prepared, by gentle pipetting, in excess of plain media. Dead cells were pelleted down by two consecutive centrifugations at 129g for 10 minutes. After each of these centrifugations, the supernatant was gently decanted into a fresh polypropylene tube and used for the next step, while the loose pellet was discarded. Live cells in the supernatant were harvested by centrifugation at 2000 x g for 8 minutes and the pellet given one wash with excess of plain media. Based on parasite counts, estimated using a haemocytometer, the pellet after the last wash was resuspended in complete media and plated at a density of 8 - 12 million cells/ mL. 1.5 – 2 billion promastigotes were routinely obtained from a single slant processed according to
the above procedure. For experimental purposes, promastigotes were directly obtained from these liquid cultures at appropriate stages of their growth cycle: log or stationary, and resuspended in appropriate volumes of complete media to get required cell densities.

3.B.1.2.c. Liquid Cultures of Axenic Amastigotes

Routine cultures of axenic amastigotes were maintained at 37°C in an atmosphere of 5% CO₂ and air, in a phosphate buffered RPMI – 1640 based medium (1 litre of RPMI-1640 with 15mM potassium chloride, 114.6mM potassium di-hydrogen phosphate, 10.38mM di-potassium phosphate trihydrate, 0.5mM magnesium chloride heptahydrate, and 24mM sodium bicarbonate, adjusted to a pH of 5.5 at 37°C) containing gentamycin at a final concentration of 1 – 1.5 mg/mL and 20% (viv) FCS (Debrabant et al., 2004).

Long-term axenic amastigotes were generated by a multi-step treatment regimen that included a few modifications to the method described previously (Debrabant et al., 2004). Briefly, live metacyclic promastigotes from 72 hour liquid cultures were harvested by the procedure described above for initiation of fresh liquid cultures. The harvested cell pellet was resuspended in 20% modified RPMI with pH adjusted to 5.5 at 26°C, and sub-cultured thrice, every 72 hours. Following the same protocol, cells were exposed to increasing temperatures of 32°C, 37°C and finally transferred to 37°C, 5% CO₂. At each new temperature, pH of the 20% modified RPMI was re-adjusted to 5.5 and cells were given three passages, with each passage after 72 hours of growth. Axenics obtained after the last transfer, were maintained like macrophage cell cultures, at 37°C in a humidified atmosphere containing 5 – 7% CO₂ in air.

Short-term axenic amastigotes were generated from metacyclic promastigotes, by a method involving a few modifications to that described previously (Somanna et al., 2002), in 10% modified DMEM by growth at 32°C for 24 hours, followed by transfer to 37°C for 24 hours. Cells obtained from the last transfer were washed, resuspended and cultured in 20% modified RPMI, with pH adjusted to 5.5 at 37°C. These axenics were maintained at 37°C, 5% CO₂ for 3 - 4 days, after which they were discarded. One variation of this procedure, resulting in lower yields, involved the transfer of promastigotes to 37°C for 24 hours followed by transfer to low pH media at the same temperature.

Since amastigotes tend to form large aggregates that interfere with plating and cell counts, just before passage or use, these clumps were disrupted by passing cell
suspensions at least five times through a 27-gauge needle, and dispersal was monitored microscopically (Goyard et. al., 2003).

3.B.1.2.d. Storage and Reactivation of Leishmania cell cultures

Leishmania donovani cells were stored for long term either as glycerol stocks or DMSO freeze downs.

To prepare glycerol stocks of the parasite, cells were obtained from mid to late exponential phase cells (OD$_{600nm}$ in the range of approximately 1.5 – 2.5, at a path-length of 1cm). 1.2mL of these cultures was mixed by repeated pipetting with 0.4mL of ice-cold sterile 80% (v/v) glycerol in appropriately labelled freezing vials chilled on ice for at least 5 minutes. These were chilled on ice for about 30 minutes, transferred to -20°C for an hour, and finally stored at -70°C. Cells from these stocks were observed to be capable of revival till at least a year after their preparation.

To reactivate these cells, frozen glycerol stocks were thawed on ice for 5 minutes. The entire contents of a vial were used to inoculate 10mL of mDMEM with 15-20% (v/v) FCS and appropriate antibiotic(s), in a ventilated cell culture flask incubated at 26°C and diluted 1:5 or 1:10 (v/v) into fresh medium during their mid growth phase. Motile parasites were usually observed almost immediately. If cells remained sluggish even 12 hours after their revival, cultures were sedimented at 2000 x g for 5 minutes, the pellet resuspended in fresh growth medium and incubated overnight under the conditions described above.

Cells in the mid to late exponential phase of growth were washed and incubated in fresh complete media at least 12 hours prior to the preparation of DMSO freeze downs. Dead cells were removed (according to the procedure described in section 3. B. 1. 2. b.). Live cells were harvested and washed thrice with complete media at 2000 x g for 8 minutes at 4°C. After the last wash, the pellet was resuspended in ice cold, sterile Solution A (3mL of media + 2mL of FCS) by repeated pipetting and 1mL of this suspension was aliquoted per freezing vial. These vials were incubated on ice for 2 minutes, after which 1mL of ice cold, sterile Solution B (4mL of FCS + 1mL of DMSO) was added per vial and mixed by gently flicking. These freeze downs were chilled at -20°C for an hour, transferred to -70°C overnight, and finally stored in liquid nitrogen. Cells from such freeze downs were observed to capable of reactivation till at least 3 years after their preparation.
To reactivate these cells, frozen DMSO freeze downs were thawed in water heated to exactly 37°C. The entire contents of a vial were gently transferred to a polypropylene tube with 50mL of plain modified DMEM with gentamycin (thawed to 26°C) and sedimented at 290 x g for 8 minutes. The supernatant was carefully decanted and the pellet washed at least thrice with plain media. In each wash, cells were resuspended in 30mL of plain media by gently flicking the tube and sedimented at 290 x g for 8 minutes. After the final wash, the pellet was gently resuspended in exactly 5mL of media with 20% (v/v) FCS and incubated at 26°C for 24 hours. If cells had not recovered motility, they were harvested and washed thrice with plain media at 290 x g for 8 minutes. These were incubated under the same conditions as described before. By 48 hours, motile cells were observed in the reactivated cultures, though most parasites remained sluggish. Healthy cultures with actively motile and dividing cells were usually obtained after 4 - 7 days of growth at 26°C.

3.B.1.3. Infections of Macrophages

For infections, macrophages obtained from 50-70% confluent cultures, typically after 72 hours of growth, were processed using the procedure described above. These macrophages were plated in white DMEM or RPMI supplemented with 10% (v/v) FCS at densities of 0.1 million cells/ well in 24-well plates; 0.8 million cells/ well or 0.5 – 0.7 million cells/ cover slip in 6-well plates. Incubation for 6-8 hours at 37°C, 5% CO₂ was allowed for adherence.

Prior to infection, the infecting promastigotes or amastigotes were washed and resuspended in the white DMEM or RPMI used for macrophage culture. Parasite loads or the multiplicity of infection (MOI) varied from 1:10, 1:25, 1:50 or 1:100. Infections were routinely performed in a total volume of 1mL/ well of complete media at 37°C, 5% CO₂. After 6 hours, free parasites were removed in two washes by serial dilution with plain white media, and a final wash with excess of ice-cold 50mM phosphate buffered saline or PBS (150mM NaCl, 50mM Na₂HPO₄. 12H₂O, 50mM NaH₂PO₄ 2H₂O in water, adjusted to a pH of 7.2 – 7.4). A sufficient volume of complete white media was added to each well and cells were incubated for 18 hours before microscopic analysis (Sudhandiran and Shaha, 2003).

Infections were visualised by staining with the cell permeant DNA dye - Syto11 (Molecular Probes, Eugene, OR). Working solutions were prepared just before use by making a one in six (v/v) dilution of the 1mM stocks of the dye in plain white media. 10µl of
this working solution was added/mL of culture media. Plates were incubated in the dark at 37°C for about 10 minutes and observed microscopically immediately after staining.

3.B.2. Biochemical and Cell Biology Techniques

3.B.2.1. Assay for Cytotoxicity by MTT Reduction

MTT \([3-(4,5\text{-dimethylthiazol-2-yl})-2,5\text{-diphenyltetrazolium bromide}]\) assay was performed by the protocol described previously (Mosmann, 1983). It is based on the ability of a mitochondrial dehydrogenase enzyme from viable and physiologically active cells to cleave the tetrazolium rings of the pale yellow MTT and form membrane impermeant dark blue formazan crystals that accumulate within these cells. Addition of a detergent to the stained cells liberates the crystals and solubilises them. The number of surviving cells is directly proportional to the levels of the formazan product, detected at an absorption maximum of 570nm.

To perform this assay, cells were harvested after appropriate treatments by centrifugation at 2000 x g for 8 minutes. The pellet was given two washes with warm RPMI-1640 without phenol red. An appropriate volume of the stock solution of the dye (5mg/mL in RPMI-1640 without phenol red) was added to the pellet to get a final concentration of 250µg MTT/mL of cell suspension. The stained cells were harvested after incubation for 2 hours at 26°C. Pellets were resuspended in residual media and solubilised with 100µl of lysis buffer (20% SDS in 50% dimethyl formamide) / reaction for a minimum of 8 hours in the dark at 37°C. Cells with the dye solution were centrifuged at 12,000 x g for 2 minutes. The supernatant was decanted and its fluorescence intensity measured in a micro-plate reader at the wavelength mentioned above.

To plot a standard curve of cell number vs. absorption at 570nm, a cell suspension of known density was serially diluted, processed by the MTT assay, and analysed for their fluorescence intensities. Using the regression equation of such a curve, results of this assay after cytotoxic treatments were expressed in terms of the number of surviving promastigotes.
3.B.2.2. Assay for Promastigote Density

Promastigote density of a cell suspension was measured as a function of its optical density at 600nm in a UV spectro-photometer. The correlation between these two parameters was established by plotting a standard curve of parasite density vs. OD 600nm.

To plot this curve, serial dilutions of a cell suspension of promastigotes at a known density, estimated using a haemocytometer, were prepared. Parasites in these standards were harvested by centrifugation at 2000 x g for 8 minutes, and resuspended in the same volume of 1X PBS. These were loaded in quartz cuvettes and their optical density measured at 600nm. A linear curve was plotted and the regression equation for this curve was determined statistically. For samples with unknown density, the regression equation derived from the standard curve was used to calculate cell numbers.

3.B.2.3. Flow Cytometry

Flow cytometry is defined as the measurement of the cellular and fluorescent properties of cells in suspension as they pass by a laser or other light source. The measurements are represented by changes in light scattered, light absorbed, and light emitted by a cell as it passes by fixed detectors directed off the light source. From these measurements, specific populations and subsets within them are defined and even isolated physically using a dedicated cell sorter typically by manipulating cell charge. Prior to this procedure, the cells have to be labelled using a specific fluorescent probe. As the cells pass through the cytometer, all light signals, whether from fluorescently labelled cells or from the beam scattered by unlabeled cells, are transferred to a computer and transformed into digital signals. These signals can then be displayed as histogram graphs or as two-dimensional graphs called dot-plots or contour-plots. In these diagrams, one parameter is plotted against another in an X versus Y axis display.

Cells from different experimental groups were run on a BD-LSR flow cytometer equipped with a 20mV, 488 nm air cooled argon-ion laser. Both Fluorescence 1 (FL1, green) and Fluorescence 2 (FL-2, red) were measured in log modes – a 530/28 band pass filter was used for the former and a 576/26 band pass filter was used for the latter. Cells were isolated from fragments by gating on the forward and side scatter signals, and then promastigotes were detected and analysed according to their relative fluorescence intensities compared with unstained promastigotes. Analyses were performed on 20,000
gated events, and numeric data were processed using WinMDI shareware. Data for all analyses was depicted as contour plots; except for that showing the extent of FL-1 scatter in GFP positive transfectants, which was depicted as a histogram. All plots are representative of at least two experiments.

3.8.2.4. Assay for Viability by Propidium Iodide Exclusion Method

The nucleic acid dye propidium iodide (PI) is excluded from viable cells with intact cell membranes but retained by non-viable cells with damaged cell membranes.

To assess viability, parasites exposed to appropriate treatments were harvested by centrifugation at 2000 x g for 5 minutes at room temperature. The pellet was washed once and finally resuspended in filtered 50mM PBS. Just before analysis, a sufficient volume of the 1mg/mL dye stock was added to this suspension to get a final concentration of 3μg/mL of PI. Staining intensities were measured flow cytometrically at 570nm.

This assay was routinely performed with parasites at densities of 10 - 20 million promastigotes/mL. All viability assays included an untreated control and a positive control. Positive controls were prepared by fixing samples using 4% formalin for 15 – 30 minutes at room temperature. Ideally, the PI positivity of untreated controls from healthy cultures never exceeded 20%, whereas those of fixed promastigotes ranged between 60 - 80%.

3.8.2.5. Assay for Promastigote Motility vs. Morphology

Assays for *Leishmania* promastigote motility and morphology were performed as described previously (Das et al., 2001).

Cell motility was checked by microscopic inspection of a promastigote suspension, at a density of 10 - 20 million cells/mL, in a Neubauer haemocytometer at different time points after exposure to heat shock at 37°C. At least 200 cells were counted in each sample, preferably from different fields. All samples were coded to avoid bias, and prepared in triplicate. Cells were scored as motile or non-motile and expressed as percentages.

Similarly, to assay for parasite morphology, cells in suspension were harvested by centrifugation at 2000 x g for 8 minutes and pellets resuspended in residual buffer. These were fixed with 4% formaldehyde (in 50mM PBS) at room temperature for 15 minutes with
end-to-end shaking. After one wash with 50mM PBS, fixed cells were loaded on glass slides and observed microscopically. For each sample, at least 200 cells were counted, preferably in different fields. Samples were prepared in triplicate and cells scored for different phenotypes.

3.8.2.6. Microscopy

Cells were visualised under a Nikon inverted fluorescence microscope C1 fitted with a scanning head (Nikon Inc., Kanagawa, Japan). For recording Nomarski images, an oil immersion 60X DIC objective in the transmission mode was used with a digital cooled CCD camera (Media Cybermetics, Silver Spring, MD). Images were analysed using the Image Pro Plus version 5.0 soft-ware (Media Cybermetics, Silver Spring, MD).

3.8.2.7. Assay for Detection of Apoptosis by Annexin V vs. PI staining

Staining for this assay was performed using the Vybrant Apoptosis Assay kit #2 as described previously (Mehta and Shaha, 2004). This was based on the principle that the human anti-coagulant Annexin V (a 35kDa calcium ion dependent phospholipid binding protein) displays a high affinity for PS. Thus, Annexin V labeled with a fluorophore binds to the PS exposed on apoptotic cells; while PI binds tightly to the nucleic acids of all non-viable cells – apoptotic or necrotic. As a result, in a cell population stained with Alexa Flour 488 Annexin V and PI, live cells show little or no fluorescence; non-viable cells show red fluorescence; and apoptotic cells show green fluorescence.

After appropriate treatments, cells were harvested and given two washes with ice-cold 50mM PBS and resuspended in freshly prepared chilled 1X Annexin binding buffer (50mM HEPES, 700mM NaCl, 12.5mM CaCl₂, pH 7.4): at 100µl per assay. The density of the promastigote suspension was adjusted between 10 - 20 million cells/mL. 5µl of the Alexa Flour 488 Annexin V and 1µl of the working solution (100µg/mL) of PI were added to each 100µl of the cell suspension. Cells were incubated at room temperature for 15 minutes. After this incubation, 400µl of 1X Annexin-Binding buffer was used to dilute out the sample. Cells were mixed gently and chilled on ice. Fluorescence intensities of the stained cells were measured flow cytometrically at 530nm and 570nm.
3.B.2.8. Assay for Detection of DNA Fragmentation

Staining for this assay was performed using the Apoptosis Detection Assay kit according to the manufacturer's instructions and as described previously (Mehta and Shaha, 2004). This kit is based on the principle that the enzyme Terminal Deoxynucleotidyl Transferase (TdT) catalytically incorporates fluorescein-12-dUTP at 3'-OH DNA ends, formed by the action of endogenous endonucleases on nuclear DNA in apoptotic cells, to form a polymeric tail that can be visualized directly by flow cytometry or fluorescence microscopy.

To perform this assay, cells were harvested after appropriate treatments and washed twice with ice cold (0.22μM filtered) 50mM PBS. The washed pellet was resuspended in residual buffer and fixed in freshly prepared 4% formaldehyde for 15 minutes at room temperature on an end-to-end shaker. After one wash with 50mM PBS to remove residual fixative, cells were permeabilised with 0.2% (v/v) Triton X-100 for 5 minutes on ice. The permeabilised cells were washed once and incubated with the equilibration buffer provided in the kit for 15 minutes on ice. Cells from this step were harvested and each reaction incubated with 44μl of buffer containing 5μl nucleotide mix (50μM Flourescein-12-dUTP, 100μM dATP, 10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 7.6) and 1μl of TdT enzyme, for 1 hour at 37°C. The reaction was terminated with 200μl of 20mM EDTA per sample. Labeled cells were harvested and analyzed for their fluorescence intensity at 530nm.

Typically, around 80 million promastigotes were used per reaction. This high initial density ensured a sufficient number of cells were analyzed even after fixation or permeabilisation induced cellular loss. Positive controls for this reaction were prepared by exposing promastigotes to heat shock at 42°C for 3 hours.

3.B.2.9. Assay for Measurement of Cytosolic Calcium

Changes in intracellular calcium concentrations were monitored with the fluorescent probe Flou-3AM (Flou-3 acetoxymerthyl ester) as described previously (Mukherjee et al., 2002). Briefly, freshly isolated cells were harvested and given two washes with Krebs buffer (118mM NaCl, 25mM NaHCO₃, 4.8mM KCl, 1.2mM KH₂PO₄, 1.2mM MgSO₄, 11mM Glucose, 1.5mM CaCl₂, 2H₂O) to remove all traces of phenol red and FCS. They were loaded with 5μM Flou-3AM, containing 1μM Pluronic Acid F-127 for
proper dispersal, in fresh buffer for an hour at 26°C. Excess dye was removed by one wash with Krebs buffer and cells were incubated at room temperature for 30 minutes in dark to allow complete hydrolysis of the dye trapped in the cytosol. At the end of this incubation, cells were given another wash and resuspended in fresh buffer to remove all traces of the non-hydrolysed dye. Fluorescence was measured at 26°C at an excitation of 488nm and emission of 522nm. At the end of each experiment, a calibration was performed to convert the fluorescence values into absolute calcium concentrations. The concentration of calcium was calculated using the equation,

$$[\text{Ca}^{2+}] = K_d \frac{(F - F_{min})}{(F_{max} - F)} \quad \text{(Eq. 1)}$$

where, $K_d$ is the dissociation constant of the $\text{Ca}^{2+}$ - Flou-3AM complex (390nM), and $F$ represents the fluorescence intensity of cells. $F_{max}$ represents the maximum fluorescence (obtained by treating cells with 1% Triton X 100), and $F_{min}$ corresponds to the minimum fluorescence (obtained by treating cells with 8mM EGTA). Fluorescence intensities were expressed as the increase in calcium concentrations with respect to base-line concentrations obtained before stimulation.

3.B.2.10. Assay for Measurement of Mitochondrial Calcium

For the separate measurement of mitochondrial calcium levels, freshly isolated cells were given two washes with Krebs buffer by centrifugation at 2000 x g for 5 minutes to remove all traces of phenol red and FCS. Washed cells were loaded with 5μM Rhod-2AM (Rhod-2 acetoxyethyl ester), containing 1μM pluronic acid F-127 for proper dispersal (Brookes et al., 2004), in fresh buffer for an hour at 26°C, according to the method described previously (Mukherjee et al., 2002). Excess dye was removed by one wash with Krebs buffer. Cells were resuspended in fresh buffer and incubated for a minimum of 30 minutes in dark to allow complete hydrolysis of the dye trapped in the mitochondria. The longer the period of this incubation, the more specific is the staining intensity a measure of the mitochondrial calcium levels. Prior to use, all traces of the non-hydrolysed dye - especially that trapped non-specifically in the cytosol - were removed by one wash with fresh buffer. Fluorescence intensities of the stained cells were measured fluorimetrically at an excitation of 530nm and emission of 576nm. To convert fluorescence values into absolute calcium concentrations, a calibration was performed at the end of each experiment. The concentration of calcium was calculated using the equation,
$[\text{Ca}^{2+}] = K_d \left[ \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)} \right] \quad \text{(Eq. 2)}$

where, $K_d$ is the dissociation constant of the $\text{Ca}^{2+}$ - Rhod-2AM complex (570nM), and $F$ represents the fluorescence intensity of cells. $F_{\text{max}}$ represents the maximum fluorescence (obtained by treating cells with 1% Triton X 100), and $F_{\text{min}}$ corresponds to the minimum fluorescence (obtained by treating cells with 8mM EGTA). Fluorescence intensities were expressed as the increase in calcium concentrations with respect to base-line concentrations obtained before stimulation.

3.B.2.11. Assay for Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential of promastigotes was measured using the mitochondrial vital cationic probe JC-1 (5, 5', 6, 6' - tetrachloro - 1, 1', 3, 3' - tetraethyl - bezimidazolylcarbocyanine chloride) according to the method described previously (Dey and Moraes, 2000) with slight modifications (Mukherjee et al., 2002). The concentration of the lipophilic JC-1 in the mitochondria is directly proportional to its potential and ATP generating capacity. At low concentrations, the dye exists as a monomer (emission 530 nm, green fluorescence); but at higher concentrations, it forms J-aggregates (emission 590 nm, red fluorescence). Thus the fluorescence ratio of the aggregates of the probe to its monomers is considered to be an indicator of the relative mitochondrial energy state of the cell.

To perform this assay, cells were harvested after appropriate treatments, resuspended in complete media, mixed with 10μM JC-1 (from 10mM stocks) and incubated in dark for 10 minutes at 37°C. Labelled cells were given two washes with complete media at 2000 x g for 5 minutes to remove excess dye; and resuspended in complete media. Fluorescence intensities of the labelled cells were measured fluorimetrically at the two wavelengths mentioned above. Mitochondrial membrane potential was expressed as the fluorescence ratio (FL ratio) of the reading at 590nm to the reading at 530nm (590:530).

Typically, this assay was performed with parasites at densities ranging between 20 - 50million promastigotes/mL.

Intracellular levels of H$_2$O$_2$ were measured using the cell permeant probe CM-H$_2$DCFDA as described previously (Mukherjee et al., 2002). This probe is a non-polar compound that readily diffuses into cells, where it is trapped by hydrolysis to the non-fluorescent derivative dichlorodihydrofluorescein. In the presence of an appropriate oxidant, dichlorodihydrofluorescein is oxidised to the highly fluorescent 2, 7 - dichlorofluorescein. The dye is optimally oxidised in the presence of hydroxyl radicals. However, the dye primarily detects H$_2$O$_2$ because of the short half-life of the hydroxyl radicals.

For this assay, harvested cells were washed twice and resuspended in Krebs Buffer at a density of 10 - 20 million promastigotes/mL. 4μg/mL of the CM-H$_2$DCFDA stock (prepared in dimethyl sulfoxide) was added per reaction. Cells were incubated with the probe for 30 minutes in the dark at room temperature with end-to-end shaking to ensure uniform staining. Stained cells were harvested at 2000 x g for 5 minutes. Excess dye was removed by one wash with Krebs buffer. Fluorescence intensities of the labelled cells were measured fluorimetrically at an excitation wavelength of 488 nm and emission wavelength of 530 nm. Basal readings were taken for 10 minutes at 26°C. Subsequently, stained cells were exposed to appropriate treatments and readings taken at appropriate intervals. For each experiment, measurements were performed in triplicate and expressed as arbitrary fluorescence intensity units (AFU).

3.B.2.13. Cell Sorting

The GFP expressing cells in transfected populations were enriched by fluorescence activated cell sorting on a BD FACS Aria equipped with a 488nm air-cooled argon laser. A 530/28-band pass filter was used for acquisition and fluorescence was measured in the log mode. The population showing highest expression of GFP was selected, sorted out and analyzed using the BD FACS Diva software.
3.B.3. Molecular Biology Techniques

3.B.3.1. Primer Design

Primers against specific stretches of the \textit{L. donovani} cHSC73 and iHSP72 genes were designed bio-informatically using the Primer3 program (http://frodo.wi.mit.edu) with sequences of the \textit{L. tarentolae} homologues (Brochu et al., 2004) as templates. Primer pairs against sequences encoding the entire HSP83 gene as well as the N-terminal and C-terminal domains of the iHSP72 gene from \textit{L. infantum} (Rico et al., 1999) were directly used to amplify counterparts of these sequences in \textit{L. donovani}. To clone the C-terminal domain of the iHSP72 gene into the \textit{Leishmania} specific expression vector pXG-GFP2', sequences of the insert - specific primer pairs were bio-informatically modified to add NotI restriction sites to the 5' and 3' flanking ends of the amplified nucleotide sequence. Details of specific primer pairs are summarised in Table 3.1.

3.B.3.2.a. Extraction of Total RNA from \textit{Leishmania donovani}

TriZOL reagent was used for extraction of total RNA from \textit{Leishmania donovani}. Briefly, \(10^9\) promastigotes were harvested by centrifugation at \(2000 \times g\) for 8 minutes and washed twice with \(50\text{mM}\) PBS to remove all traces of media and FCS. The pellet was resuspended in residual buffer and lysed in 2mL of ice-cold TriZOL reagent. The lysates was homogenised by repeated pipetting (approximately 50-100 strokes were given). Cell debris, polysaccharides and high molecular weight DNA were pelleted down by centrifugation at 12,000 \(x\) \(g\) for 10 minutes at 4\(^\circ\)C. The supernatant was gently decanted into a fresh microcentrifuge tube and 200\(\mu\)L of chloroform \(\text{mL}\) of TriZOL was added to it by vigorous shaking. The mixture was incubated at room temperature for 2 - 3 minutes before centrifugation at 12,000 \(x\) \(g\) for 15 minutes at 4\(^\circ\)C. This separated the mixture into a lower organic phase and an upper aqueous phase. The aqueous phase containing the RNA and forming about 60\% of the total volume was decanted into a fresh tube. This was mixed with 500\(\mu\)L of isopropanol/ mL of TriZOL reagent and incubated for at least 10 minutes at room temperature. The RNA was precipitated out in the form of a loose pellet by centrifugation at 12,000 \(x\) \(g\) for 10 minutes at 4\(^\circ\)C. After the supernatant was discarded, the pellet was washed with 75\% ethanol in 0.1\% (v/v) DEPC-treated water; air- dried for about 10 minutes at room temperature and re-dissolved in DEPC treated water for storage and
<table>
<thead>
<tr>
<th>Serial Number</th>
<th>Name of the L. donovani Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cHSC73</td>
<td>5’ – TCT CTG AGG GTG TAC AGG AC - 3’</td>
<td>5’ – CAC TTT CTT CAT CGC TTT CT - 3’</td>
</tr>
<tr>
<td>2</td>
<td>iHSP72</td>
<td>5’ – GTC CGA AGA GAA CAC ATA CG - 3’</td>
<td>5’ – AAG CTT TTA GTC GAC CTC CT - 3’</td>
</tr>
<tr>
<td>3</td>
<td>HSP83</td>
<td>5’ – GGG AAT TCA TGA CGG AGA CGT TCG CGT T – 3’</td>
<td>5’ – GGA AGC TTT CAG TCC ACC TGC TCC ATG C – 3’</td>
</tr>
<tr>
<td>4</td>
<td>iHSP72 N-terminal domain</td>
<td>5’ – GCG GAT CCA CAT TCG AAG GCC CCA TC - 3’</td>
<td>5’ – CGA GCT CTT GCC GCC CGT CAG GAT GAA - 3’</td>
</tr>
<tr>
<td>5</td>
<td>iHSP72 C-terminal domain</td>
<td>5’ – GCG GAT CCA GCA AGC AGA CGG AGG GC - 3’</td>
<td>5’ – GGA AGC TTT TAG TCG ACC TCC TCG ACC TTG G - 3’</td>
</tr>
<tr>
<td>6</td>
<td>Notl added to iHSP72 C-terminal domain</td>
<td>5’ - GC GGC CGC CCA CCA TTA GTC GAC CTC CTC GAC - 3’</td>
<td>5’ - GC GGC CGC TGC GGA TCC AGC AAG CAG ACG G - 3’</td>
</tr>
<tr>
<td>7</td>
<td>Poly A Binding Protein</td>
<td>5’ – CCA AGG ACG AGG GCA CAC – 3’</td>
<td>5’ – CGC CGA CAA AGA TGG AGG T – 3’</td>
</tr>
</tbody>
</table>

Table 3.1. Sequences of primers used in the current study. Restriction sites for Notl are highlighted in bold.
use. The purity (ideal if the A$_{260}$/A$_{280}$ ratio is greater than 1.7) and concentration of these preparations were estimated by spectroscopy. These preparations were preferably used fresh but could be stored in highly concentrated forms at -70°C for up to a month with negligible degradation.

3.B.3.2.b. Reverse Transcription of the Total cellular RNA into cDNA

Since RNA preparations were not suitable for polymerase chain reactions, complementary DNA sequences (cDNA) were synthesised using reverse transcription. Reverse transcription for total cellular cDNA synthesis from *L. donovani* was performed with the Superscript II First Strand Synthesis kit as per the manufacturer’s protocol. In brief, 5µg total RNA was denatured at 65°C for 5 minutes in the presence of Oligo dT$_{16}$ and dNTPs and incubated at 42°C for another 2 minutes with DTT, MgCl$_2$ and RNAseOUT in 10X Reverse Transcriptase Buffer. 1µl/reaction of the Superscript Reverse Transcriptase enzyme was added to the denatured RNA and incubated at 42°C for 50 minutes. The enzyme was denatured by heating to 70°C for 15 minutes. The reaction was collected by a quick high-speed centrifugation and the complementary RNA strand degraded by incubating with RNaseH for 20 minutes at 37°C. The purity (ideal if the A$_{260}$/A$_{280}$ ratio is greater than 1.6) and concentration of the cDNA preparation obtained as a result was analysed by UV-spectroscopy. These preparations could be stored in highly concentrated forms for much longer periods at -70°C with negligible degradation.

3.B.3.3. Preparation of Genomic DNA

Genomic DNA preparations of *L. donovani* were obtained using the GenElute™ Mammalian Genomic DNA Miniprep kit. Briefly, 100 million parasites were harvested by centrifugation at 2000 x g for 8 minutes, washed twice with 50mM PBS to remove all traces of media and FCS, and mixed by repeated pipetting with 200µl of the resuspension solution provided in the kit. 20µl of RNaseA solution was added and the suspension incubated for 2 minutes at room temperature. Cells were vigorously vortexed for 15 seconds after addition of 200µl of Lysis Solution C and 20µl of Proteinase K, and incubated at 70°C for 10 minutes to ensure complete homogenisation. Pre-assembled GenElute MiniPrep Binding Columns were equilibrated with 500µl of Column Preparation solution by centrifugation at 12,000 x g for 1 minute. After 200µl of 95 - 100% ethanol was
mixed with the lysate by vortexing for 5 - 10 seconds, the entire volume was transferred to
the binding columns and centrifuged at 6500 x g for 1 minute. The column was washed
twice, each wash with 500μl of wash buffer, by centrifugation at 6500 x g for 3 minutes. To
collect the genomic DNA, the column was incubated at room temperature for 5 minutes
with 200μl of elution buffer and centrifuged at 6500 x g for 5 minutes. The purity and
concentration of this preparation was estimated by UV spectroscopy and the genomic DNA
stored at -20°C.

3.8.3.4. Estimation of purity and concentration of RNA and DNA preparations

The purity and concentration of RNA or DNA preparations were analysed by
spectroscopy. To do this, 2μL of the RNA or DNA preparations were used to make 1:500
dilutions in RNase free water, mixed by repeated pipetting and transferred to quartz
cuvettes. Their absorption was estimated at two wavelengths: 260nm and 280nm. Prior to
these readings, the spectrophotometer was calibrated to zero using salt-free water, used
for making the RNA or DNA dilutions, as a test probe.

The purity of either preparation was calculated as the ratio of the \( A_{260}/A_{280} \) readings. The concentration of these preparations was estimated by the following
calculation:

\[
[RNA \text{ or } DNA] \text{ in ng/μl} = A_{260} \times \text{constant} \times \text{Dilution factor} \quad (\text{Eq. 3})
\]

where, the constant = 40 for RNA and 50 for DNA, to denote the fact that an RNA
concentration of 40mg/mL or a DNA concentration of 50mg/mL corresponds to an optical
density of 1.0; and the dilution factor for a 1:500 dilution of RNA in water would be 500. For
each preparation, concentration was calculated as the mean value of at least two
independent readings.

3.8.3.5. Polymerase Chain Reaction

Polymerase chain reactions (PCR) were used to amplify specific nucleotide
sequences from L.donovani using either genomic DNA or total parasite cDNA as
templates. All cloning reactions consisted of an initial denaturation at 94°C for 4 - 10
minutes (10 minutes were preferred for colony PCR's); 20 - 30 cycles of denaturation at
94°C for 60 seconds, annealing at primer specific temperatures for 90 seconds, extension
at 68 - 72°C for 120 seconds (68°C was preferred for all High Fidelity Taq Polymerase mediated reactions whereas 72°C was preferred for all Taq Polymerase catalysed reactions); and final extension at 68 - 72°C for 15 minutes.

Relative expression levels of specific gene(s) in samples exposed to differential treatments were determined by semi-quantitative PCR's. The optimal number of cycles for specific primer pairs used in such reactions varied, and was defined as the minimal number required for linear amplification of serial dilutions of cDNA preparations from control or untreated parasite samples. Equal concentrations of the DNA from all samples were then used in amplifications for the number of cycles determined during standardisations, and visualised by agarose gel electrophoresis. Primer pairs against housekeeping genes, like the Poly A binding protein, were used as loading controls in amplifications with the same template DNA concentrations from all samples.

3.B.3.6. Agarose Gel Electrophoresis for DNA Separation

DNA fragments were resolved, based on their expected sizes, on 0.8 - 2% agarose gels, containing 0.5µg/mL of ethidium bromide in Tris-Acetate EDTA or TAE buffer (40mM Tris-acetate, 2mM EDTA, pH 8.1). An appropriate volume of gel loading dye, containing bromo-phenol blue, was added to each sample prior to loading. Routine gels were run at 80-100V; those for gel elution were run at 40 - 60V. The position of DNA in the samples was determined relative to the position of the tracking dye (bromo-phenol blue) present in all samples. Resolved DNA bands were visualized in a UV trans-illuminator at 312nm. Relative band sizes were determined with respect to bands of specific DNA markers run in parallel.

3.B.3.7. Elution of DNA from Agarose Gels

The DNA to be eluted from agarose gels were prepared in gel loading dye (according to the protocol described in section 3.B.3.5.). These were loaded on 1% low melting point or LMP agarose gels, cast with preparative combs, and run at 40V.

The resolved bands were visualised under a UV-transilluminator, quickly (to minimise exposure to UV) excised from the gel using a clean sharp scalpel and placed in previously weighed 1.5mL microcentrifuge tubes. The sizes of the gel slices were
minimized by removing excess agarose. Filled tubes were weighed again and the pure weights of the gel slices were calculated by subtraction. DNA was eluted using the QIAquick gel extraction kit, according to the manufacturer's instructions. Briefly, three volumes of Buffer QG were added to 1 volume of gel (100 mg – 100 μl) and the tubes were incubated at 50°C for a minimum of 10 minutes, until gel slices had completely dissolved. To help dissolve gel, tubes were vortexed every 2 – 3 min during the incubation. To bind the DNA in the dissolved gel, the sample was applied in 700μl aliquots to the binding columns provided and centrifuged at 12,000 x g for 2 minutes. After each centrifugation, flow-through was discarded. Once the entire sample had been loaded, all traces of agarose were removed by loading the columns with 500μl of QG buffer and centrifugation at 12,000 x g for 2 minutes. The column was washed with 700μl of PE buffer by centrifugation at 12,000 x g for 2 minutes. After the flow through from this wash was discarded, the column was centrifuged again at the same speed for 2 minutes to remove all the residual ethanol from the PE buffer. The DNA was eluted out into a clean microcentrifuge tube with a minimal volume (10 – 20μl) of sterile water by centrifugation at 12,000 x g for 5 minutes after an incubation of 5 minutes at room temperature. The purity and concentration of the eluted DNA was determined either by agarose gel electrophoresis or UV spectrophotometry. The eluted DNA was stored at -20°C.

3.8.3.8. Sub-cloning PCR Products into pGEM-TEasy Vector

Sub-cloning eluted DNA bands into the commercially available pGEM-TEasy vector, following the manufacturer's instructions, allowed sequencing and efficient digestion. Briefly, 3μl of eluted 1μg/μl DNA was ligated with 1μl of the provided stock of pGEM-TEasy vector in the presence of 1μl of T4 DNA Ligase in a 10μl reaction volume. This was incubated overnight at 4°C or left at room temperature for an hour. 8μl of the ligation mix collected by a single high speed spin was used for transformation. 100μl of each transformation mix was plated on thawed blue-white selection plates (LB agar plates containing ampicillin and 40μl/ plate of the Blue-white select reagent) and incubated at 37°C for 12 hours to allow growth. White colonies were screened for the presence of the desired gene.
3.B.3.9. Screening of Bacterial Transformants

Bacterial colonies obtained by transformation were checked for the presence of either the plasmid or a gene inserted into the plasmid by a PCR based or restriction digestion based screening. Briefly, all the transformed colonies were numbered, picked from their original plate and used to inoculate a single fresh LB plate with ampicillin, called the master plate.

For a PCR based screening, the master mix for all the PCR reactions to be performed was made and stored on ice. The volume of autoclaved water for each reaction (approximately 15μl for every 25μl reaction) was aliquoted into PCR tubes. Individual colonies from the master plate were picked with autoclaved 10μl pipette tips or toothpicks and flicked in this volume of water. The master mix was equally aliquoted out into each tube. This screening master mix consisted of a set of primers that amplified a specific fragment of known size. The first step of all such screening PCR reactions was incubation at 94°C for not less than 10 minutes to ensure complete cellular lysis and release of DNA. Results of the polymerase chain reaction were run on an agarose gel and positive colonies were identified by comparison with appropriate positive and negative controls. This method was preferred for transformations involving ligation of a desired insert into the transforming vector. Thus, primers were typically designed such that the amplified PCR product obtained for screening contained a part of the plasmid backbone with a part of the desired gene sequence. This increased specificity of the reaction and prevented interference with the amplified product from a gene endogenous to the bacteria.

For a restriction digestion based screening, individual colonies were picked like in the previous method and used to inoculate primary cultures of 3mL of LB media (20g LB broth base in 1 liter of water) with ampicillin at a final concentration of 100μg/mL. After 8 hours of growth at 37°C, these were used to inoculate secondary cultures. The plasmid from secondary cultures was extracted using MiniPrep kits (the procedure for which is described in section 3. B. 3. 9). The concentration and purity of the plasmid was determined by UV spectro-photometry and subjected to specifically designed screening restriction digestions. The products of the digestion were analyzed on an agarose gel and positive colonies were identified based on their pattern of fragment formation. Both positive and negative restriction screens were employed to identify the presence of a specific insert. This increased specificity by clearly identifying colonies as those containing the vector alone from those containing the vector with the desired insert.
3.B.3.10. Storage and Reactivation of Bacterial cells

Transformed bacterial colonies positive for the recombinant vector were stored as glycerol stocks, and reactivated when required.

Glycerol stocks were prepared from well-grown secondary cultures of bacterial cells (obtained by the procedure described in section 3.B.3.8). Freezing vials, appropriately labeled, were chilled on ice for 5 minutes. 250μl of ice cold, sterile 50% (v/v) glycerol was aliquoted into these vials and mixed completely by repeated pipetting with 750μl of bacterial cell suspension. These were chilled slowly for 30 minutes on ice, transferred to -20°C for an hour, and finally stored at -70°C.

Cells from these stocks were observed to capable of revival for at least two years from their preparation. To reactivate the stored cells, glycerol stocks were thawed on ice for 5 minutes. The freezing vials were gently flicked to mix uniformly. Contents of an entire glycerol stock were resuspended in 3mL of LB media, preferably without antibiotic, and incubated at 37°C, 250 rpm for 1 - 8 hours. Cells from these cultures were streaked on LB plates with or without a selection antibiotic. The plates were incubated for 12 - 16 hours at 37°C for individual colonies to appear. Alternatively, a flamed bacterial loop was used to directly streak cells from thawed glycerol stocks on LB plates, incubated under conditions described above.

3.B.3.11. Plasmid Preparation

Plasmid DNA was purified by the alkaline lysis method. This formed the principle for Qiagen kits for plasmid purification. Briefly, cells from an appropriate volume of overnight bacterial culture were harvested by centrifugation and then re-suspended in P1 buffer [50mM Tris-HCl (pH: 8.0), 10mM EDTA, 100μg/μl RNase A]. The resuspended cells were lysed by addition of P2 buffer [200mM sodium hydroxide and 1% SDS] and incubated at room temperature for 3-5 minutes. The plasmid DNA was re-natured by adding neutralizing P3 buffer [3M Potassium Acetate (pH: 5.5)], which precipitated out the genomic DNA and cellular proteins. The plasmid DNA obtained in supernatant, after centrifugation at 12,000 x g for 10 minutes, was precipitated by addition of 0.7 volume of Isopropanol. The pellet obtained by another centrifugation was washed with cold 70% ethanol to remove salt impurities. The plasmid was air-dried and re-suspended in 10mM Tris-HCl (pH: 8.0) at -20°C for further use.
3.8.3.12. **Restriction Digestion**

Restriction enzymes are endonucleases that recognize specific base sequences of a DNA double helix and hydrolyse in specific positions. This leads to the cleavage of the initial DNA sequence in two or more pieces. Digestion of plasmid DNA with specific restriction endonucleases was typically performed as 50µl reactions composed of 5µl of the Enzyme Specific Reaction Buffer, 2µl (approximately 10 - 20 units) of Restriction Enzyme and 2 - 5µg of DNA in autoclaved water. In general, 10 units of restriction endonuclease are sufficient to overcome variability in DNA source, quantity and purity. The reaction was mixed thoroughly by repeated pipetting and collected by a single high speed spin. Routine digestions were carried out at 37°C for 1 hour. Preparative digestions were carried out under similar conditions but in larger volumes, for 3 hours. Double digestions were performed sequentially. To do this, the restriction product of the first digestion was run on an agarose gel. The desired restriction product was eluted out and used as the DNA source for the second digestion.

3.8.3.13. **Preparation of competent cells**

Chemically competent bacterial cells of DH5α and XL-1BLUE strains of *E. coli* were prepared by the calcium chloride method. Briefly, 100mL of LB media was inoculated with 1% inoculum from a well grown overnight bacterial culture incubated in a 37°C incubator-shaker until an OD<sub>600</sub> of exactly 0.5 (1cm path-length) was reached. Excessive growth was prevented by incubating this flask on ice for 10 minutes. Cells were harvested by centrifugation at 2000 x g for 15 minutes at 4°C. The pellet was gently resuspended in 20mL of ice cold 100mM CaCl<sub>2</sub> and incubated on ice for at least 20 minutes before centrifugation at 2000 x g for 15 minutes at 4°C. Cells were resuspended in 4mL of ice-cold, sterile 85mM CaCl<sub>2</sub> in 15% glycerol (w/v), aliquoted and stored at -80°C for further use.

3.8.3.14. **Transformation of chemically competent *E. coli* cells with plasmid DNA**

To transform, 5-8µl of the purified plasmid or a ligation mix of the plasmid with the desired gene, collected by a single high speed spin, was gently mixed with 50µl of thawed competent DH5α or XL-1BLUE *E. coli* cells. After 20 minutes on ice, cells were given a heat
shock at 42°C for exactly 45 seconds. These were plunged in ice for 5 minutes to allow recuperation from shock. 950μl of SOC medium (0.5% yeast extract, 2% tryptone, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, and 10mM MgSO₄ dissolved in water, autoclaved and cooled, before adding 20mM glucose) was added to the cells and incubated at 37°C for 90 minutes with shaking at 250 rpm. An appropriate volume of the transformation mix was evenly spread on thawed LB-agar plates with ampicillin. The plates were air dried and incubated at 37°C for 12 - 18 hours. The colonies obtained were screened for the presence of either the desired plasmid or gene.

3.8.3.15. Transfection of *Leishmania donovani*

3.8.3.15.a. DNA Preparation

Recombinant vector DNA (pXG-GFP2' + iHSP72 C-terminal domain) was obtained by ligation of cloned *Leishmania donovani* iHSP72 C-terminal domain with the NotI digested pXG-GFP2' plasmid, a *Leishmania* specific expression vector, derived from pXG (Ha *et al.*, 1996), and kindly provided by Dr. Stephen M. Beverley (Washington University, St. Louis, MO). It was prepared from transformed XL-1Blue cells using the EndoFree MegaPrep kit. The quality of the DNA preparation was analysed by both agarose gel electrophoresis (high quality preparations show a very high intensity band of super coiled DNA, a much fainter band for circular DNA and almost no linear DNA fraction) and UV spectrophotometry (ideally the purity of the plasmid preparation should be 1.8 or above).

3.8.3.15.b. Processing of *Leishmania*

A 72 - hour liquid culture (initiated by the protocol described in section 3. B. 1. 2. b.) of promastigotes was used to inoculate 40mL of complete 10% modified DMEM at a dilution of 1:10. Parasites in early exponential phase, ideal for transfections, were obtained after 24 hours of growth of the freshly inoculated cultures at 26°C. Observed microscopically, they showed a predominance of tear-drop shaped cells that were motile and actively dividing with little or no debris. Once dead cells were removed from pooled cultures by the protocol mentioned above, live cells were harvested and given two washes with ice-cold cytomix buffer (120mM KCl, 0.15mM CaCl₂, 10mM KH₂PO₄, 25mM HEPES,
2mM EDTA and 2mM MgCl₂, pH 7.6) at 340 x g for 8 minutes at 4°C. After the final wash, they were counted and resuspended at a density of 100 million cells/mL of buffer.

3.B.3.15.c. Electroporation

For a single electroporation, 20 - 30µg of plasmid DNA (in water or 10mM Tris pH 8.0) was transferred to a fresh microcentrifuge tube. 400µl of chilled cell suspension, at the required density of 100 million cells/mL of cytomix buffer, was transferred to the tube and mixed with the DNA by gentle tapping. The entire mixture was gently transferred to a 0.4mm electroporation cuvette (BTX, San Diego, CA) and left on ice for 10 minutes. Air bubbles between the electrodes were avoided. Electroporation was performed using a modified high voltage protocol as described previously (Coburn et al., 1991; Ha et al., 1996; LeBowitz et al., 1990). To do this, cells were pulsed twice at 1500V and 25µF (exponential decay protocol) in a BioRad gene Pulser X cell. A gap of at least 10 seconds was given between the first and second pulse and the time taken for each pulse was read and documented (usually between 7-9 milliseconds). Pulsing normally resulted in foaming on the surface of the cell suspension. After the second pulse, the cuvette was returned to ice for exactly 10 minutes. A gap of at least 1 minute was given before the next round of electroporations to allow the regeneration of the condensator. The cell suspension was transferred to exactly 5mL of 20% medium 199, pre-incubated at 26°C for an hour, in ventilated cell culture plates. These were allowed to recover at 26°C for 12-16 hours without gentamycin or antibiotics for selection.

3.B.3.15.d. Selection of Transgenics

After the first 12 hours at 26°C, electroporated cultures were observed microscopically every four hours to check for recovery of motility. This was important to prevent non-recombinant parasite subpopulations from outgrowing the transfected cells. Typically, cells were observed to be ready for selection by 16 hours after electroporation. At this stage, cells were harvested, from 1 or 2mL of the 5mL electroporated culture, by centrifugation at 2000 x g for 5 minutes. These cells were resuspended in residual media and a maximum of 50-100µl of this suspension was spread (with a flame sterilised bacterial spreader) on freshly poured M199 selection plates (7.5mL of cooled 20% 2X M199 with 20µg/mL of gentamicin sulphate or G418 and without gentamycin + 7.5mL of
melted 2% bacteriological agar). The plates were sealed with Parafilm® (Chicago, IL) and incubated at 26°C for 7 - 14 days. Individual colonies were picked and used to inoculate 1mL of 20% M199 with 20μg/mL of G418. These were passaged every 48 hours, with each passage involving an increment of the G418 selection pressure to a final concentration of 1mg/mL. Once recombinant clones had been identified and sorted for enrichment of the GFP-positive population, these cultures were routinely maintained either on slants or in liquid cultures at a selection pressure of 200μg/mL of G418.

3.B.3.16. DNA sequencing

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

3.B.4. Protein Biology Protocols

3.B.4.1. Preparation of Whole Cell Lysates for Protein Analysis

After appropriate treatments, cells were harvested by centrifugation at 2000 x g for 8 minutes, washed twice with 50mM PBS to remove all traces of media and FCS, and resuspended in residual buffer. A minimal volume of 2X sample buffer (62.5mM Tris HCl pH 6.8, 10% v/v Glycerol, 1% w/v SDS, 1% v/v mercaptoethanol, 0.01% w/v bromo-phenol blue), thawed at 99°C for 5 minutes, was added in drops to the pellet with vigorous vortexing to ensure complete lysis. The lysate was denatured by boiling at 99°C for 15 minutes and precipitates pelleted down by centrifugation at 13,000 x g for 5 minutes. The supernatant was decanted into fresh tubes and stored at -20°C. Just before use, these were thawed at 99°C for 5 minutes and collected by centrifugation at 13,000 x g for 5 minutes. Preferably, whole cell lysates were always prepared fresh. Stored lysates were preferably used within a week after their preparation.
3.8.4.2. Estimation of Total Protein Concentration in Whole Cell Lysates

Total Protein concentration of whole cell lysates was performed using the CB-X Protein Assay kit, by a modification of the Bradford method (Bradford, 1976). In brief, 1mL of chilled (-20°C) precipitant solution was added to 10µl of the whole cell lysate and centrifuged at 13,000 x g for 10 minutes. The supernatant was discarded and the pellet air-dried at room temperature for 5 minutes before 50µl each of the solubilising reagents A and B were added to it. Samples were vortexed vigorously to completely dissolve the pellet and incubated at room temperature for 5 minutes after adding 1mL of colour reagent. These were read spectro-photometrically at 595nm. A standard curve of the OD595 plotted against different concentrations of Bovine Serum Albumin (BSA) was used to determine concentrations of samples from their absorbance at the wavelength mentioned above.

3.8.4.3. Electrophoresis for Protein Separation

Electrophoresis for protein separation was performed using the Laemmli buffer system (Laemmli, 1970) on 10% or 12.5% polyacrylamide gels under reducing or denaturing conditions (SDS-PAGE). Buffers used to prepare the gels were 30% acrylamide solution (30% acrylamide, 2.7% bis-acrylamide), 4X running gel buffer (1.5M Tris HCl, pH 8.8), 4X stacking gel buffer (0.5M Tris HCl, pH 6.8), 10% SDS and 10% ammonium persulphate. The thickness of these gels varied between 1 - 1.5mm depending upon the purpose for which they were run. To visualize protein bands on gels by Coomassie staining, 1mm gels were preferred for their higher resolution. To transfer separated protein bands to membranes for Western blots, 1.5mm gels were preferred for their greater loading volumes. A discontinuous gradient gel system was used. Gels were equilibrated and run in tank buffer (25mM Tris Base, 192mM Glycine, 0.1% w/v SDS) at 40 – 60V when the sample was in stacking gel and at 80 – 100V once it entered the resolving gel. A broad range molecular weight marker RPN756 or RPN800 was loaded in parallel to the protein samples to evaluate the approximate molecular weights of the resolved proteins.

3.8.4.4. Visualization of Protein bands on Polyacrylamide Gels

Polyacrylamide gels were stained using Imperial Protein Stain (Pierce, Rockford, IL) containing the colloidal form of Coomassie Blue, according to the manufacturer’s instructions. Briefly, the gel(s) were washed thrice with de-ionised water, each wash for 5
minutes at room temperature, with shaking. A sufficient volume of the Imperial Protein Stain was added to the gel(s) to immerse them completely. The gel(s) were incubated with the stain for an hour at room temperature, with shaking. At the end of this period, the stain was discarded and the gel(s) given two quick washes with de-ionised water. To de-stain completely, the gel(s) were immersed in a sufficient volume of de-ionised water at room temperature for an hour on shaker.

3.B.4.5. Transfer of Proteins onto Nitrocellulose Membranes

Protein transfer from gels to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) was performed at 40V for 2.5 hours at room temperature in chilled transfer buffer (192mM glycine, 25mM Tris base pH 8.3, 20% v/v methanol). Protein bands on nitrocellulose membranes were visualized by napthol black staining to determine efficiency of transfer.

3.B.4.6. Visualisation of Transferred Proteins on Nitrocellulose Membranes

Nitrocellulose membranes were washed once with 50mM PBS for 10 minutes at room temperature to remove all traces of transfer buffer. These were stained in naphthol blue black (in 7% acetic acid) for a maximum of 30 seconds at room temperature. To destain, these membranes were given three consecutive washes, each wash for 10 minutes at room temperature, with excess of 7% acetic acid.

3.B.4.7. Western Blots

Western blot analysis was performed as described previously (Aravinda et al., 1995) with slight modifications. After staining, membranes were given two washes with wash buffer or PBS-T (50mM PBS containing 0.1% Tween-20), each wash for 5 minutes at room temperature, to remove all traces of the acetic acid. Non-specific sites were blocked with 5% ECL blocking reagent (prepared in wash buffer) by incubation for an hour at room temperature on a shaker at 40 rpm; or with 2% ECL blocking reagent by incubation at 4°C overnight. Excess of the blocking reagent was removed by three washes, each for 20 minutes, at room temperature on a shaker set at 60 – 80 rpm. Appropriate primary antibody dilutions (usually at 1:1000) were prepared either in 0.1% PBS-T or 0.1% PBS-T with 1%
ECL Blocking Reagent. Blots were incubated with the primary antibody dilutions for 2 hours at room temperature on a shaker set at 30 - 40 rpm, or overnight at 4°C. Unbound antibody was removed by two washes with 0.1% PBS-T, each wash for 30 minutes at room temperature on a shaker set at 60 - 80 rpm. Secondary antibody dilutions, usually ranging from 1:2,000 to 1:10,000, were always prepared fresh in 0.1% PBS-T. Blots were incubated with these dilutions for exactly an hour at room temperature, on a shaker set at 30 - 40 rpm. Unbound antibody was removed by two washes with 0.1% PBS-T, each wash for 15 minutes at room temperature on a shaker set at 60 - 80 rpm. These membranes were given a final wash with 50mM PBS for 15 minutes at room temperature before developing to visualize bands.

3.8.4.8. Detection by Enhanced Chemi-Luminescence or ECL

Immuno-blotted protein bands were detected on nitrocellulose membranes using the ECL reagents from the EZ-ECL kit, according to the kit manufacturer's instructions. Enhanced chemi-luminescence is based on the ability of horseradish peroxidase (HRP) to catalyse the oxidation of cyclic diacylhydrazides such as luminal, in the presence of hydrogen peroxide. The oxidized luminal formed immediately after this reaction is in an excited state. On decay to its ground state, it emits light that can be enhanced in the presence of phenolic compounds. This allows detection of specific antigens immobilized on nitrocellulose membranes and bound to HRP - labeled antibodies/streptavidin.

Briefly, equal volumes of the EZ-ECL solutions A and B were mixed to obtain sufficient solution to cover membranes (0.1mL/cm²) after their final wash with 50mM PBS. The mixture was equilibrated for at least 5 minutes at room temperature. Excess buffer was drained off from the washed blot(s) without allowing excessive drying out of the membrane. These blots were incubated with the detection mix, directly added to the membrane (protein side up), for 1 - 3 minutes at room temperature. Once the excess detection mix was drained out, membranes were enclosed in Saran wrap and air pockets gently smoothened out. These blots were placed protein side up in an X-ray film cassette. All subsequent steps were performed only under red safety lights. A sheet of X-ray film was placed on the blot and the cassette closed and exposed. Unless already standardized, the first exposure was usually for 15 - 30 seconds. At the end of this period, the exposed film was transferred to developer, and a new film placed in the cassette for exposure. As soon
as bands became visible on the exposed film, it was washed with water. These were incubated in fixer for 1 - 2 minutes at room temperature. Excess of fixer was gently washed off with running water and the film air-dried. The length of exposure of the second film was determined according to the intensity of the signal in the first film.

3.B.4.9. Denistometry

Densitometry was performed using the ImageJ 1.33u software (http://rsb.info.nih.gov/ij/) designed by Wayne Rasband (National institute of Mental Health, Bethesda, Maryland).

3.B.4.10. Statistics

Paired comparisons were conducted using the Student's t-test. Significance was assumed for values of $P < 0.05$. Results are expressed as mean ± SE.

3.C. Instrumentation

Megatech BOD Incubator was obtained from MegaTech International (Bombay, India). CO$_2$ incubators were purchased from Heraeus (Allerod, Denmark). Laminar Flow Hoods were procured from Kartos International, Ltd. (New Delhi, India).

Optiphot fluorescence microscope, Nikon E600W upright fluorescence microscope and Nikon confocal microscope C1 were from Nikon (Tokyo, Japan).

Eppendorf Centrifuge 5810R was procured from Eppendorf (Hamburg, Germany) and Biofuge Fresco table - top Centrifuge was procured from Heraeus (Allerod, Denmark).

LS50B Luminescence Spectrometer was purchased from PE Biosystems (Norwalk, CT). FlouStar Optima multi-plate fluorescence reader was purchased from BMG Lab Technologies Inc. (Offenburg, Germany). UV-160A UV-visible
spectrophotometer was from Shimadzu (Tokyo, Japan). uQuant Microplate Reader was procured from Biotek Instruments, Inc. (Winooski, VT).

Submarine DNA electrophoresis system was purchased from Bangalore Genie (Bangalore, Karnataka, India). Protean-II polyacrylamide gel system and Mini Transblot Cell were procured from Bio-Rad Laboratories (Hercules, CA).

GenePulsar Xcell electroporator was procured from Bio-Rad Systems (Milan, Italy). BD-LSR and BD FACSARia flow cytometers were purchased from BD Biosciences (San Jose, CA).

MultiTemp III water bath and Electrophoresis Power Supply EPS 500/400 were purchased from Pharmacia Biotech-AB (Uppsala, Sweden). Gyratory water Bath Shaker was procured from New Brunswick Scientific Co., Inc. (Edison, NJ).

Peltier Thermal Cycler-200 was purchased from MJ Research (Waltham, MA). Doc-It System was procured from UVP Bio Imaging Systems Incorporation (Upland, CA).

3.0 Bibliography


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