Leishmania cell surface prohibitin: role in host–parasite interaction

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Introduction

The study of host–parasite interaction has attracted considerable attention in recent years because of the realization that much needs to be identified at the level of host–parasite contact to provide a proper understanding of successful pathogenesis. Since the initial interaction between host and the pathogen is dependent on cell surface-associated molecules in both organisms, characterization of surface linked proteins and glycoproteins have been of significant interest (Kedzierski et al., 2004). Leishmania spp., the parasites that cause leishmaniasis in vertebrates, use host macrophages to propagate infection, and binding between the parasite and the host cell is mediated by complementary molecules on the parasite and the host surface. Among a number of parasite molecules that are implicated in binding to the host, the most studied are the surface localized LPG and gp63 (Matlashewski, 2001). The mammalian host molecules implicated in parasite binding include, Fc, CR3, mannose-fucose receptor, Toll-like receptor, CD11b and CD35 (Blackwell et al., 1985; Wilson and Pearson, 1986; Guy and Belosevic, 1993; Rosenthal et al., 1996). Leishmania presents itself in four distinct clinical forms; cutaneous, mucocutaneous, visceral (VL) and post kala-azar dermal leishmaniasis (Handman and Bullen, 2002). VL caused by the Leishmania donovani-infantum cluster is a systemic disease, which is fatal if left untreated. There are an estimated 500,000 new cases of VL (Desjeux, 2004) and in developing nations, the overlap of endemic regions of VL with regions of HIV infection poses very serious threat (Alvar et al., 2008) and has become a major challenge to the control of VL (Hailu and Berhe, 2002). One important advancement towards the development of successful treatment of parasitic diseases caused by the kinetoplastid parasites is the completion of the genome sequencing projects of multiple kinetoplastid parasites causing a variety of diseases (Ivens et al., 2005). This has opened up opportunities to validate structure–function association of various molecules through relevant experiments.

Leishmania spp. parasites have a digenic life cycle where the disease is transmitted to the vertebrate host by infective metacyclic promastigotes residing in the pharynx of the invertebrate vector (Mukherjee et al., 2002). Therefore, molecules selectively upregulated in metacyclic promastigotes could be associated with changes prior to...
or during host–parasite interaction. This hypothesis along with the report of increased expression of an evolutionarily conserved orthologue of mammalian prohibitin in the metacyclic promastigotes (Almeida et al., 2004) provided an interesting opportunity to look at a protein that possibly functions during macrophage–parasite interactions. In Trypanosoma brucei, prohibitin is reported to be present in the flagella (Broadhead et al., 2006) and is upregulated during ConA induced apoptosis in the same parasite (Welburn and Murphy, 1998). Plasma membrane localization of prohibitin has been reported in T. brucei (Broadhead et al., 2006; Bridges et al., 2008), a closely related species to Leishmania. A very recent report indicates a mitochondria linked function in Trypanosoma brucei (Tyc et al., 2009). Barring these few reports on identification, no literature is available on prohibitin function in the kinetoplastid parasites.

In higher eukaryotes, prohibitin is involved in events like cell proliferation (Joshi et al., 2003), ageing (Coates et al., 2001), apoptosis (Joshi et al., 2003), B-cell maturation (Woodlock et al., 2001) and the maintenance of mitochondrial integrity (Nijtmans et al., 2000). Prohibitin can also act as a tumour suppressor protein (Wang et al., 2002), serve as mitochondrial chaperones or play a role in mitochondrial biogenesis (Artal-Sanz et al., 2003). Apart from its presence in the mitochondria of higher eukaryotes, mammalian intestinal epithelial cells were reported to use surface prohibitin to bind to Vi polysaccharide of Salmonella typhi (Sharma and Qadri, 2004).

Utilizing various approaches, we provide evidence that prohibitin is expressed on the promastigote surface particularly concentrated at the aflagellar pole and the flagellar pocket, flagellar pocket being a site for exocytosis and aflagellar pole being the region of initial contact between the host and the parasite. The protein is GPI anchored to the Leishmania surface, and mutants with a substitution of amino acid at the GPI-link site expressing lesser surface protein were unable to bind to macrophages as efficiently as the wild-type protein overexpressing cells. Presence of anti-prohibitin antibodies during macrophage–Leishmania interaction in vitro produces lesser infection. Evidence shows that the cognate binding partner for Leishmania prohibitin on the macrophage is surface HSP70. Presence of anti-prohibitin antibodies in VL patients shows that Leishmania donovani prohibitin is able to generate a strong humoral response in humans.

Results

Prohibitin is concentrated at the aflagellar pole of promastigotes

To establish the identity of Leishmania donovani prohibitin, whole-cell lysates of Leishmania promastigotes were obtained at the log phase of growth using an in vitro model that mimics the conversion of procyclic promastigotes to metacyclics within the sandfly vector (Sacks, 1989). A mammalian anti-prohibitin antibody recognized a single band at 30 kDa (Fig. 1A, lane b). Subsequently, the same antibody was used to immunoprecipitate the protein from log phase promastigote lysates (Fig. 1A, lane c). MALDI-TOF analysis of tryptic digests of the immunoprecipitated protein described above showed signature sequences of L. major prohibitin (Fig. S1A and B) that confirmed the specificity of the antibody and identity of the Leishmania donovani protein. It was evident from the Western blot that Leishmania prohibitin shared similar molecular weight of 30 kDa as that of its mammalian orthologue. Immunolocalization studies using live cells at 4°C were carried out to identify the presence of any surface protein on the promastigotes. Prohibitin was localized to two discrete locations on the cell surface, at the tip of the aflagellar pole and at the flagellar pocket (Fig. 1B, c, d and f). Flagellar pocket localization was confirmed by colocalization of red staining of FM 4–64, a styryl dye that labels the flagellar pocket (Mullin et al., 2001) with the green FITC label of prohibitin staining (Fig. 1B, b, d and f). Mask of colocalization delineating the exact overlapping areas of colocalization is shown in Fig 1B, e. Antibody preincubated with recombinant prohibitin could not significantly stain cell surface (Fig. 1C, c and d) as compared with anti-prohibitin antibody staining only (Fig. 1C, a and b). This showed specificity of the prohibitin immunostain. Figure 1D shows the three dimensional reconstruction of the z-sections from confocal imaging of cells immunostained for prohibitin in viable conditions shown in Fig. S2A, a–i. The above data clearly indicated presence of prohibitin on promastigote surface at two poles of the cell. Figure 1E shows evaluation of prohibitin staining by flow cytometry demonstrating increased surface prohibitin expression in metacyclics as compared with procyclies where the two populations were separated using peanut agglutinin (PNA).

Permeabilized cells stained to show intracellular protein demonstrated discrete localization of prohibitin in vesicular structures but not in the nucleus (Fig. S2B, a–e). There was a minor overlap of prohibitin staining with Mitotracker Red, a dye that is specific for mitochondria, in the region of the kinetoplast (Fig. S2B, e). The immunostained vesicular structures were not endoplasmic reticulum (Fig. S2C, a–d) or lysosomes (Fig. S2C, e–h) as ER-Tracker Blue or LysoTracker Red, respectively, did not stain the vesicles. Immunoelectron microscopy confirmed the light microscopic data, as gold-labelled secondary antibody was detected at the tip of the promastigote surface (Fig. 2A, b), control stained with unrelated antibody shows the absence of any label (Fig. 2A, a). Observations of surface stained intact cells demonstrated
**Fig. 1.** Characterization of *L. donovani* prohibitin.

A. Western blots of *Leishmania* total cell lysate (lane b) and immunoprecipitate obtained with anti-prohibitin antibody from the total cell lysate (lane c) probed with anti-prohibitin antibody shows immunoreactivity in the region of 30 kDa. Lane a, molecular weight marker.

B. Immunolocalization of prohibitin. a, Nomarski image of b–f; b, cells showing red staining of flagellar pocket with FM4-64 dye; c, immunoreactive prohibitin detected with secondary antibody conjugated to FITC at two different poles of the cells; d, colocalization of prohibitin stain and FM4-64 labelling; e, mask of colocalization; f, overlap of a, b and c. Scale is 5 μm. FP, flagellar pocket; AP, aflagellar pole, PR, prohibitin.

C. Specificity studies with live cells; a, Nomarski image of b; b, immunoreactive prohibitin as detected by anti-prohibitin antibody; c, Nomarski image of d; d, staining of cells with anti-prohibitin antibody preincubated with recombinant prohibitin prior to staining. Note the significant reduction of staining in d. Scale is 5 μm. FP, flagellar pocket; AP, aflagellar pole, PR, prohibitin.

D. Three-dimensional reconstruction of optical sections (1 μm each of 8 sections) by confocal imaging shown in Fig. S2A demonstrating the nucleus (N) and the kinetoplast (K) as blue spots stained with Hoechst 33342 and the green spots are prohibitin staining at the aflagellar pole (AP) and the flagellar pocket (FP).

E. Flow cytometric analysis of the isolated populations of viable metacyclic and procyclic promastigotes immunostained with anti-prohibitin antibody, showing higher surface staining intensity in metacyclics as compared with procyclics. The table represents the mean fluorescence intensity (MFI) for each group and the fold increase in staining intensity. Data are ± SEM (*n* = 3). *P* < 0.05, procyclic versus metacyclic.

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Fig. 2. Ultrastructural localization of *L. donovani* prohibitin.

A. Immunoelectron micrograph of promastigotes labelled for prohibitin with secondary antibody conjugated to gold (18 nm). a, ultrathin section of sample stained with control antibody only; b, ultrathin section of a promastigote stained with prohibitin antibody followed by secondary antibody conjugated to gold showing localization of the gold particles on the membrane; c, intact cell stained with unrelated primary antibody; d, intact cell stained with prohibitin antibody followed by secondary antibody showing concentration of the gold particles at the aflagellar end. Red arrows point to gold particles. AP, aflagellar pole.

B. a, schematic diagram of flagellar pocket (McGwire et al., 2002) showing the localization of proteins destined for the surface; b, Ultra-thin section through the region of the flagellar pocket of *L. donovani* promastigote immunostained with an unrelated primary antibody; c, Ultra-thin section through the region of the flagellar pocket of *L. donovani* promastigote immunostained for prohibitin followed by secondary antibody conjugated to gold (18 nm) showing localization of the gold particles within the lumen of the flagellar pocket (FP). Red arrows indicates gold particles. d, intact cells stained with an unrelated primary antibody followed by secondary antibody conjugated to gold (18 nm) showing absence of gold particles; e, intact cells stained as above with anti-prohibitin antibody showing prohibitin localization in the flagellar pocket. F, flagella; K, kinetoplast; M, mitochondria, FP, flagellar pocket.

Adapted from McGwire et al, 2002 (J. Biol. Chem. 277:8802)
concentration of gold label at the aflagellar pole extending to about 2 μm in a cell of 10–12 μm (Fig. 2A, d). Figure 2A, c is a control cell labelled with unrelated antibody. Figure 2B shows localization in the flagellar pocket. Localization of the protein at the flagellar pocket could be due to the traffic of GPI-linked proteins destined for the surface that are released from the flagellar pocket (McGwire et al., 2002). Figure 2B, a shows a schematic diagram of the process (McGwire et al., 2002). Figure 2B, b is a control cell labelled with unrelated antibody showing lack of staining and Fig. 2B, c shows the gold label representing prohibitin stain confined to the flagellar pocket lumen that has a plasma membrane continuous with the surface (Fig. 2B, a) and on the cell membrane surrounding the flagellar pocket. Observations of surface stained intact cells demonstrated concentration of gold label at the flagellar pocket (Fig. 2B, e). Figure 2B, d is a control cell labelled with unrelated antibody. These data confirmed the localization pattern obtained with light microscopy.

The *Leishmania donovani* prohibitin gene was cloned as a 807 bp nucleotide fragment (GenBank™ accession number DQ246217) (Fig. 3A, lane a) using primers designed from the *L. major* prohibitin nucleotide sequence (LmjF16.1610, GeneDB gene database of *L. major*). Bioinformatic analysis of the derived protein sequence predicts the presence of a signal sequence at the N-terminus with a probable cleavage site between positions 23–24. The prohibitin domain was predicted between amino acids 23–214 and the presence of a GPI anchor site at position 252 (Fig. 3B). The derived amino acid sequence was aligned with other sequences using Clustal W as hosted by the European Bioinformatics Institute, UK. Prohibitin domain was shared with other kinetoplastid parasites like *T. cruzi*, *T. brucei*, *L. major*, *L. infantum*, malarial parasite and mammals (Fig. S3). The phylogram generated from the alignment demonstrates good conservation within the groups with *Leishmania*, *Trypanosoma*, *Plasmodium* and the mammals forming separate clusters. The phylogenetic distance between the mammalian, *Arabidopsis thaliana* and *Leishmania* prohibitin sequences was substantially large while the distance with the *Trypanosoma* was also significant, with the genus *Leishmania* diverging as a separate cluster (Fig. 3C).

**Prohibitin is GPI anchored to the parasite surface**

As shown above, the prediction of a GPI anchor site within the prohibitin sequence and prior knowledge that majority of *Leishmania* surface proteins with diverse functions like gp63 and LPGs are attached to the membrane through a GPI anchor (Ilgoutz and McConville, 2001) prompted us to explore the possibility of *Leishmania* prohibitin being linked to the surface by a GPI anchor. For this, promastigotes were scanned for the presence of surface prohibitin after treatment with PIPLC, an enzyme that cleaves GPI linked proteins (Sundler et al., 1978). Since PIPLC treatment requires to be carried out at 37°C, first, the status of prohibitin in the cells after 1 h of exposure to 37°C was checked. This temperature is physiological for the parasite because 37°C is the host body temperature and the parasites are at this temperature during host–parasite interaction. A significant increase in surface prohibitin was noted at 37°C (Fig. 4A, lane b) as compared with cells retained at 22°C (Fig. 4A, lane a). As evident from the flow cytometry data shown in Fig. 4B, PIPLC treatment resulted in a notable reduction in surface prohibitin staining of cells. Microscopic observations confirm the reduction of surface prohibitin staining from PIPLC-treated cells at 37°C (Fig. 4C, c and d) as compared with untreated cells at 37°C (Fig. 4C, a and b). The above data clearly demonstrated that prohibitin was GPI anchored to the parasite surface. When PIPLC-treated cells denuded of surface prohibitin were allowed to recuperate at 22°C and the status of surface prohibitin probed at different time points through live cell staining, it was evident that storable prohibitin appeared on the surface in the form of small aggregates (red arrows) near the flagellar pole that eventually accumulated at the aflagellar pole (Fig. 4C, e–j). Figure 4D shows a flow cytometric analysis of cells from the above experiment, demonstrating that surface repopulation checked at 4 h (magenta line) was almost equivalent to control levels (black line) while 2 h recovery cells (blue line) showed a positive shift as compared with only PIPLC-treated cells (green line). MFI indicates the value of changes.

**Modulation of surface prohibitin affects infection of macrophages in vitro**

Working on the hypothesis that prohibitin could be essential for host–parasite interactions because of the demonstrated increase of prohibitin in infective metacyclics and its localization at the pole of the cell that interacts with the macrophage (Courret et al., 2002), we tested the effects of overexpression of prohibitin in host–parasite interactions. Prohibitin was overexpressed in promastigotes in two forms; one was the overexpression of the wild-type protein and another was the overexpression of a protein with a mutation at the GPI anchor site (by replacing aspartic acid 252 to aspartic acid at the N-glycosylation site of the prohibitin gene, N252D) so that translocation of GFP–prohibitin fusion protein on the surface is compromised. Figure 5A provides evidence of fused GFP–prohibitin expression by amplification of the GFP–prohibitin mRNA from cells transfected with wild-type and the mutant prohibitin (N252D) using primers covering...
sequences from GFP as well as prohibitin (Fig. 5A, lane b and c) in respective stable cell lines. Western blots of total cell extracts probed with anti-prohibitin antibody from promastigotes overexpressing the wild-type protein (Fig. 5B, lane c) and mutant GFP–prohibitin N252D (Fig. 5B, lane d) confirms expression of recombinant proteins expressed at higher molecular weights. The surface expression of the GFP–prohibitin protein in transfected cells was confirmed by Western blots of membrane preparation from cells expressing GFP–prohibitin showing a reactive band at 60 kDa (Fig. 5C, i, lane b), which is not present in membrane preparations of cells transfected with only GFP (Fig. 5C, i, lane a) or GFP–prohibitin–N252D (Fig. 5C, i, lane c). Figure 5C, ii shows results of FACS scan of membrane ghosts (cell bodies devoid of intracellular contents) of transfected cells where an increase in expression of GFP–prohibitin (as a measure of GFP fluorescence) on the surface was observed in GFP–prohibitin transfected cells and a comparatively lower expression in the N252D mutants (as a measure of

Fig. 3. Cloning and domain analysis of *L. donovani* prohibitin.
A. Agarose gel (1.5%) showing amplified fragment of full length *L. donovani* prohibitin gene from genomic DNA using primers designed against GeneDB annotated *L. major* prohibitin sequence (LmjF16.1610). m, marker; lane a, amplified 807 bp prohibitin fragment.
B. Protein sequence based schematic representation of putative domains of prohibitin analysed using NCBI Blast, Target P, Phobius and Big PI prediction softwares. Note the presence of a signal sequence spanning 1–21 amino acids at the N-terminus and GPI-anchor site at amino acid position 252 and prohibitin domain extending between amino acids 23–214.
C. A phylogram generated from the sequences analysed by Clustal W shown in Fig. S3, showing the distances between *Leishmania* spp., *Trypanosoma* spp., *Plasmodium* spp., *A. thaliana* and mammalian prohibitin, that form separate clusters. The similarities of *L. donovani* prohibitin with prohibitin from other species are indicated in percentages.
A

Prohibitin staining at different temperatures:
- 30kDa at 22°C (a)
- 30kDa at 37°C (b)

B

Table of MFI and Fold change:

| Groups                        | MFI  | Fold change |
|-------------------------------|------|-------------|
| Unstained                     | 03 ± 1 | N.A.        |
| Control                       | 19 ± 3 | 1.0         |
| 1 h at 37°C                   | 34 ± 5 | 1.8         |
| 1 h at 37°C + PIPLC           | 11 ± 2* | 0.6         |

C

Prohibitin staining in different groups:
- a, b: Unstained
- c, d: Control
- e, f: PIPLC treated

D

Prohibitin staining in recovery groups:
- a, b: Unstained
- c, d: Control
- e, f: PIPLC treated
- g, h: 2 h recovery
- i, j: 4 h recovery

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Prohibitin binds to macrophage cell surface heat shock protein 70

The above data provided evidence for prohibitin binding to macrophages, but the binding partner on the host was not known. Therefore, to determine the identity of the binding partner for Leishmania surface prohibitin on macrophages, prohibitin was synthesized in vitro using Leishmania prohibitin DNA in a transcription–translation coupled rabbit reticulocyte system where macrophage membrane preparation was added post prohibitin synthesis to the total mixture and subjected to immunoprecipitation with anti-prohibitin antibody (Fig. 7A, lane c). As a control, parallel reactions were carried out in the absence of the DNA encoding prohibitin (Fig. 7A, lane b). As is evident from the silver stained gel in Fig. 7A, there was a 70 kDa protein that was pulled down by anti-prohibitin antibody from the mixture that contained prohibitin DNA. MALDI-TOF analysis of tryptic digests of the 70 kDa protein showed the presence of mammalian HSP70 signature (Fig. 7B). Western blots of the above immunoprecipitated samples probed with anti-prohibitin antibody (Fig. 7C, lane d) and anti-HSP70 antibody (Fig. 7D, lane d) shows presence of both prohibitin and HSP70 in the same sample of the immunoprecipitate. Since the binding protein for Leishmania in macrophage membrane preparations appeared to be HSP70, possible localization of HSP70 on macrophage surface was explored to actually confirm the presence of HSP70 on the surface. Live J774 cells stained with anti-HSP70 antibody at 4°C shows distinct staining of macrophage surface HSP70 as visualized by FACS scan (Fig. S4A) and fluorescence microscopy (Fig. S4B). The surface localization of HSP70 was further confirmed by immunoelectron microscopy that showed clear localization of gold tagged secondary antibody recognizing primary anti-HSP70 antibody in J774 cells (Fig. S5B), but no such reactivity was observed in isotype antibody controls (Fig. S5A). Mouse peritoneal macrophages (Fig. S4C) and human peripheral blood monocyte-derived macrophages (Fig. S4D) stained...
Prohibitin

60kDa

30kDa

45kDa Tubulin

GFP-Prohibitin

GFP

Actin

D252N

GFP Vector

transfected

GFP-Prohibitin Vector

transfected

GFP-Prohibitin N252D Vector

transfected

Control

pXG-GFP

pXG-GFP-Prohibitin

pXG-GFP-Prohibitin N252D

% infection

0
10
20
30
40
50
60
70

3 (h)                         6 (h)

MN

P

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positive for surface HSP70 expression confirm the existence of surface HSP70 on normal macrophages. Colocalization studies carried out with antibodies against a surface macrophage marker CD-11b and anti-HSP70 antibody clearly demonstrate colocalization of the two staining on the surface of live J774 cells (Fig. S6, A) and murine peritoneal macrophages (Fig. S6, B).

To further confirm that prohibitin binds to macrophage HSP70 on the extracellular side of the macrophage membrane, live macrophages were incubated at 4°C with biotinylated recombinant prohibitin followed by labelling of the biotinylated probe with streptavidin-phycoerythrin (Fig. 7E, h). These macrophages were stained with anti-HSP70 antibody followed by labelling with secondary anti-Prohibitin antibody. The results are shown in Fig. 7E, h.

Fig. 5. Overexpression of L. donovani prohibitin.
A. Expression of GFP–prohibitin protein as seen by mRNA expression in electroporated cells after G418 selection; RNA from; lane a, vector transfected cells; lane b, GFP–prohibitin transfected cells; lane c, GFP–prohibitin N252D transfected cells amplified with primers spanning GFP and Prohibitin (GFP–Prohibitin), GFP only (GFP) and actin.
B. Western blot of lysates of transfected cells probed with anti-prohibitin antibody showing the expression of GFP–prohibitin fusion protein in pXG–GFP–prohibitin (lane c), pXG–GFP–prohibitin N252D (lane d) as a band at 60 kDa as opposed to endogenous prohibitin at 30 kDa. Lanes a and b are untransfected and pXG–GFP2 + transfected cells respectively.
C. i. Western blot of membrane preparation of GFP–prohibitin transfected cells as compared with vector transfected or N252D mutant cells probed with anti-prohibitin antibody. ii. Flow cytometric analysis of the above membranes showing GFP fluorescence only in cells transfected with GFP–prohibitin. The table represents mean fluorescence intensity for each group. Data are ±SEM (n = 3). GFP transfected (red line); GFP–prohibitin transfected (black line) and GFP–prohibitin N252D transfected (green line).
D. Results of immunocytochemistry of transfected cells with anti–GFP antibodies; d, GFP–vector transfected cells; e, GFP–prohibitin vector transfected cells; f, GFP–prohibitin N252D vector transfected cells. a,b,c being the nomarski of above respectively. Scale is 5 μm.
E. Bar graph showing percent infection of cells transfected with the above mentioned constructs at 3 and 6 h; *P < 0.05, pXG–GFP vs pXG–GFP–prohibitin; **P < 0.05, pXG–GFP–prohibitin as compared with pXG–GFP–prohibitin–N252D. Data are ±SEM (n = 3).
F. Photomicrographs of infection data at 6 h shown in E; a, untransfected; b, GFP transfected cells; c, GFP–prohibitin transfected cells; d, GFP–prohibitin N252D transfected cells. Scale is 5 μm. Note the reduced infection in N252D vector transfected cells. MN, macrophage nucleus; P, parasite nucleus.

Fig. 6. Antibody induced neutralization and infective abilities of the parasite. Flow cytometric analysis of binding of PKH green labelled L. donovani cells after treatment with Fab fragments of anti-prohibitin antibody to macrophages. The percentages in parenthesis represent the percent binding by the labelled parasites; (A) 15 min; (B) 30 min; (C) 60 min; (D) graph representing infectivity index of PKH labelled parasites treated with or without the Fab fragment of anti-prohibitin antibody.
E. coli heat shock protein 70 [Mus musculus]

MSKGPAVIDLGTTYSVCGVFQHKGVEI1ANDQGRRTTPSYVAFTD
TERLIGDAAKNQVAMNPNTVDARLRILGRFDDAVQVQSDMNHPF
MVVDAGRPKQVEYKGETKSYFPEEVSMLTKMKEIAEAYLGKT
VTNAVTVTVPAYFNSQRAKTDAGTIAGLNVLRIINEPTAAIAYG
LDKVKGAERNVLIFDLGGTFDVSSLITIEDIGFEVKSTAGDTHLG
EDFTINRMVHFIAEFKRHKKDISQNRAVRVRRTACERAKRTLS
STQASIEIDSYGIDFYSITTRAERFELNADLFEGTLDPVEKAR
DALKRQHIDILVLDGSTR1PKIQKLLHDFFNDSKLMK5NIPDEA
VAYGAAVQAAILSGKSEQVQDLVLLCLVTPLSSLGILAGNTVL
KNRTTIPHTQTQTFYTSNDQPVGLIQYVEGERAMTKDNLLGKF
EL/GIPPAIPRQRFQIEVTFIDANGILNVSAVDSKTGKRENKITND
KRKLSEKDIERNVQEAQKIAEDEKQRDKVSSKNSLEYAFNMKAT
VEDEKLQGKINDEDKQKILDKCNEIISWLDKNQ/IABKEEBEHQQKE
LEKVCNPIIITLYQSAGGMPGMGFPGGAGGAPPSSGGASGPTIEEE

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Fig. 7. Macrophage surface protein binding to *Leishmania* prohibitin.
A. Silver stained SDS-PAGE gel of the immunoprecipitates obtained from a reaction mixture of purified macrophage membranes and rabbit reticulocyte lysate with (lane c) or without prohibitin encoding DNA (lane b) showing the presence of a 70 kDa band in the reaction mixture containing the prohibitin encoding DNA (indicated by arrow). Lane a, molecular weight marker.
B. Mammalian HSP70 sequence showing underlined groups as sequences of peptides obtained by tryptic digest (underlined regions) generated from the 70 kDa band shown in (A). They are identified to share similarity with mammalian HSP70 sequence as obtained from the NCBI-BLAST.
C. Western blots of the total reaction input (lanes a and b), immunoprecipitates obtained with anti-prohibitin antibody (lanes c and d) from a reaction mixture of purified macrophage membranes and rabbit reticulocyte lysate with (+DNA) or without (-DNA) prohibitin encoding DNA probed with anti-prohibitin antibody showing the presence of prohibitin in the mixture containing the prohibitin DNA (lane d). Arrow indicates prohibitin band.
D. Western blots of the total reaction input (lanes a and b) and immunoprecipitates obtained with anti-prohibitin antibody (lanes c and d) from a reaction mixture of purified macrophage membranes and rabbit reticulocyte lysate with (+DNA) or without (-DNA) prohibitin encoding DNA (lanes d and c) probed with anti-HSP70 antibody showing the presence of HSP70 (indicated by arrow) in the immunoprecipitate containing prohibitin encoding DNA.
E. a–e, Photomicrographs of immunostained viable macrophages with anti-HSP70 antibody (g) or secondary antibody only (b) showing the presence of HSP70 on the macrophage surface. h, shows binding of biotinylated prohibitin detected with streptavidin-phycoerythrin; i, streptavidin only control; j, merge of g and h; j, mask of colocalization, a, nomarski image of b-e; f, nomarski image of g-j.

body conjugated to FITC (Fig. 7E, g). Colocalization of phycoerythrin and FITC, as shown in Fig. 7E, i, clearly shows areas where the two stainings overlap, suggesting binding between the recombinant prohibitin and macrophage surface HSP70. Figure 7E, j shows the mask of colocalization delineating areas where both stains are in close apposition.

Since the above data indicated that *Leishmania* promastigotes were binding to macrophage surface through HSP70, we downregulated macrophage surface HSP70 through siRNA transfection and checked the efficiency of binding of *Leishmania* to the macrophage. Figure 8A shows the transfection efficiency of control siRNA in macrophages and Fig. 8B shows moderate downregulation of surface HSP70 after transfection with the HSP70 siRNAs. The siRNA mixture used in this experiment was designed against four different HSP70 genes. The low knock-down efficiency despite high siRNA transfection could be due to the presence of multiple HSP70 genes being expressed and the siRNAs designed for the four genes could only be targeting a fraction of the HSPs expressed, and thereby this moderate downregulation could only be achieved. Figure 8C shows a 15% reduction in the efficiency of binding of *Leishmania* to macrophages in which siRNA has been used to downregulate the HSPs. Figure 8D shows photomicrograph of *Leishmania* attachment to host cells in the groups shown in Fig. 8C, where it is apparent that the number of macrophages interacting with the parasites is fewer in the HSP70 siRNA transfected group (Fig. 8D, d) as compared with the group transfected with scrambled siRNAs (Fig. 8D, b).

*Sera of visceral Leishmaniasis patients show the presence of anti-prohibitin antibodies*

Several *Leishmania* proteins have been identified as antigens generating antibodies in VL patients (Houghton *et al.*, 1998) that makes them important for the study of immune response to prohibitin and as possible candidates for diagnostic markers. Therefore, sera from VL patients were checked for the presence of anti-prohibitin antibodies. Anti-prohibitin antibodies were detected by an ELISA, where the identifying antigen was a peptide designed from an immunodominant region on *Leishmania* prohibitin not shared with mammals (Fig. S7A) or the malarial parasite (Fig. S7B). The antisera used were from patients whose disease profile was characterized by standardized assays for leishmaniasis (Fig. S8). Reactivity of the peptide antigen to the sera was significant in all 40 samples tested (Fig. 9A), for which a statistically significant linear regression between peptide-specific binding and patient serology was obtained (Fig. 9B). This part of the data therefore showed that *Leishmania* prohibitin was able to elicit antibodies in VL patients.

**Discussion**

Effective invasion and survival strategies are required to be developed by the kinetoplastid parasites in order to survive in two disparate climates of the poikilothermic invertebrate insect vector and the homeothermic vertebrate mammalian host. During its life cycle in the sand-fly, *Leishmania* undergoes important biochemical changes during conversion from less infective procyclic promastigotes to metacyclics with higher infectivity (metacyclogenesis) (Sacks and Perkins, 1984) and it is during metacyclogenesis that prohibitin mRNA is upregulated in the parasite (Almeida *et al.*, 2004). This makes prohibitin a potentially important candidate as a participant in host–parasite interactions. Surface molecular changes occurring in the virulent metacyclic promastigotes (Santos *et al.*, 2006) are particularly important as they are present at the interface of communication with the host cells. After transfer from the sand-fly to the vertebrate host, the life cycle demands adaptation to a change of temperature from 22 to 37°C (Mukherjee *et al.*, 2002) and several proteins involved in host–parasite interactions are upregulated at host body temperature (Handman and

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Bullen, 2002). Therefore, in our studies, the observed prohibitin increase in metacyclics as well as at 37°C reinforced the idea that prohibitin could be involved in events leading to host–parasite interactions.

Since *Leishmania donovani* prohibitin was highly conserved within the genus but was relatively distant from the human prohibitin, the two proteins being functionally different was a possibility. Also, lack of significant mito-
chondrial localization of prohibitin in the promastigotes unlike the higher eukaryotes, where the protein is localized in the mitochondria serving as a mitochondrial chaperone (Artal-Sanz et al., 2003), strongly indicated a hitherto unknown function for Leishmania prohibitin. Although a very recent report indicates that prohibitin is associated with mitochondria of Trypanosoma brucei (Tyc et al., 2009), our studies showed minimal localization in the mitochondria. Interestingly, in L. donovani, prohibitin was attached to the surface through a GPI anchor, a situation unknown for other prohibitins, however, a substantial number of Leishmania surface proteins are linked to the cell surface through GPI anchors (Ilgoutz and McConville, 2001). It was of much interest that prohibitin localization was concentrated to the aflagellar pole of the metacyclic promastigotes because these cells are known to bind to macrophages via this region (Rittig and Bogdan, 2000; Courret et al., 2002). This peculiar localization was unlike that of other molecules implicated in host–Leishmania binding like GP63 and LPG that are distributed all over the cell surface (Zhang et al., 2003). It is possible that when the parasite interacts with the host, GP63 or LPG and as yet unidentified components will cluster at a few small areas during uptake of Leishmania

Fig. 9. Prohibitin peptide-specific binding of sera from active VL patients.
A. Reactivity of VL patient sera in an ELISA to the peptide designed from a unique sequence stretch in L. donovani as shown in Fig. S6.
B. Results of linear regression analysis of peptide specific binding of patient sera from 40 VL patients. The resulting $R^2$ value is shown adjacent to the regression line.
HSP70 interacted with macrophage HSP70 verified that macrophage surface mastigotes with macrophages with partial knock-down of macrophage surface and the decreased binding of pro-interactions. The binding of recombinant prohibitin to where this interaction could be modulating signalling of macrophage surface HSP70 as parasite Leishmania identified as participation in host–parasite interactions.

Furthermore, the inhibition of parasite binding to prohibitin shows a totally different function in Leishmania unlike the higher eukaryotes. It serves as an important entity in host–parasite interactions and can be viewed as a target for drugs or a diagnostic marker.

Experimental procedures

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and Superscript First Strand Synthesis kit were purchased from Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-prohibitin antibody and rabbit polyclonal anti-α-tubulin antibody were obtained from Neomarker (Fremont, CA, USA). Anti-HSP70 antibody was obtained from Assay design (Ann Arbor, MI, USA). Alexa flour-Fluorescein isothiocyanate conjugated antibodies, Mitotracker Red, Lysotracker Red, ER tracker blue, Syto green 11 and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR, USA) and horse radish peroxidase conjugated secondary antibodies were procured from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Western blotting reagents and
enhanced chemiluminescence kit were from Amersham Life-sciences (Piscataway, NJ, USA). CB-X™ protein assay kit was obtained from G-Biosciences (Maryland heights, MO, USA). 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, USA). Reagents for PCR like Taq polymerase and dNTP mixture were obtained from New England Biolabs (Beverly, MA, USA). TNT coupled rabbit reticulocyte system and pGEM-T easy vector kit were obtained from Promega Corporation (Madison, WI, USA). Phosphorydilinositol phospholipase C (PI-PLC), PKH67 green fluorescent cell linker kit for general cell membrane labelling, papain, PNA, medium-199 (M199) and all other chemicals unless otherwise mentioned were obtained from Sigma-Aldrich Chemical (St Louis, MO, USA). 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, USA.

**Cells**

Murine J774A.1 macrophages were maintained in DMEM supplemented with 10% FBS. *Leishmania donovani* promastigotes were grown on blood agar slants as described previously (Mukherjee et al., 2002) and were transferred to Medium 199 supplemented with 10% FBS before experiments.

**Procyclic–metacyclic separation, PKH labelling and Infection**

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

Labelling of the cell membrane with the PKH67 dye was performed as per the manufacturer’s protocol; briefly, 10⁷ log stage promastigotes were incubated with PKH-67 dye (10⁻¹ M) in 1 ml diluent buffer for 5 min and the staining was subsequently quenched by addition of equal volumes of FBS. Washed cells were cultured overnight in M-199 containing 10% FBS.

J774A.1 macrophages grown to confluency were coincubated with promastigotes and macrophages

**Preparation of membranes from *L. donovani* promastigotes and macrophages**

Promastigote membrane preparation was performed using the protocol described by Snapp and Landfear (1997) with modifications. Briefly, 2 x 10⁷ cells were washed in ice-cold PBS and centrifuged at 1000 g. The pellet of the cells was resuspended in MME (10 mM MOPS, pH 6.9, 0.1 mM EGTA, 1 mM MgSO₄, 0.1% Triton X-100 and protease inhibitor cocktail) and incubated on ice for 10 min followed by centrifugation at 3000 g (Model 5810R, Eppendorf AG, Hamburg, Germany). The pellet was washed with PBS and lysed in SDS sample buffer.

Macrophages were swelled in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5) and homogenized with a Dounce homogenizer. The homogenate was stabilized by addition of stabilization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 7.5) and sequentially centrifuged at 1300 g for 15 min, 17 000 g for 15 min (Model 5810R, Eppendorf AG, Hamburg, Germany) and 100 000 g in an ultracentrifuge (Optima XL-100K, Beckman) for 1 h to obtain the membrane fraction as a pellet and the supernatant as the cytosol.

**Immunoprecipitation and protein microsequencing**

Promastigote lysates prepared by sonication in immunoprecipitation buffer (0.025 M Tris, 0.15 M NaCl, pH 7.2) were incubated with mouse monoclonal anti-prohibitin antibody (200 μg) and immunoprecipitated with Seize Primary Immunoprecipitation kit following the manufacturer’s protocol. Tryptic digests of excised bands were analysed at W.M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia, VA, USA). Sequest search algorithm (Bioworks software, Thermoelectron Corporation, Waltham, MA, USA) was used to determine cross-correlation scores between acquired spectra and the *Leishmania major* database (GeneDB, Sanger Institute, UK).

**Immunocytochemistry and flow cytometry**

Immunostaining of prohibitin was performed on fixed cells (2% formaldehyde) and live cells (stained at 4°C) using anti-prohibitin antibody (1:50) followed by Alexa Flou 488 conjugated secondary antibody at a dilution of 1:100. Fixed cells were permeabilized with 0.1% saponin during antibody incubations. Mitotracker Red (0.5 μM/10⁵ cells), LysoTracker Red (1 μM/10⁵ cells) and ER-Tracker Blue (1 μM/10⁵ cells) was used to visualize mitochondria, lysosomes and endoplasmic reticulum, respectively, while FM-464 (10 μM/10⁶ cells) was used to stain the flagellar pocket membranes of live cells stained for prohibitin. Nuclei and kinetoplast were stained by Hoechst 33342. Live cell staining for surface HSP70 was performed by incubating live macrophages with anti-HSP70 antibody (1:50) and secondary antibody (1:100). Cells were visualized and photographed as described previously (Iyer et al., 2008). Confocal microscopy was performed using Zeiss LSM 510 microscope using a 63X/1.4 oil immersion objective, the cells were simultaneously illuminated with 488 nm (laser power: 9.8%) and two photon laser set at 770 nm (laser power: 16.1%) for visualization of the green fluorescence of prohibitin staining and blue fluorescence for Hoechst 33342 respectively. Pinhole was set at 200 μm for 488 nm and 1000 μm for 770 nm laser, serial Z-sections were captured at 1 μm interval with a total stack size of 8 μm.
**Electron microscopy**

For electron microscopy, stationary phase cultures were harvested and fixed (0.8% glutaraldehyde and 4% paraformaldehyde) for 4 h at 4°C. Following wash and dehydration the samples were embedded using LR White (TAAB, Berkshire, UK). Leica Ultracut (Leica, Wetzlar, Germany) was used prepare 100 nm thin sections and processed for immunostaining using anti-prohibitin antibody (1:50) and gold (18 nm) labelled anti-mouse secondary antibody (1:20) at room temperature. To confirm the specificity of staining, unrelated antibody was used as a control at similar dilutions. The immunostained sections were viewed using Morgagni 269(D) Transmission Electron Microscope, FEI Company (Hillsboro, OR).

For electron microscopy of surface immunostained intact *Leishmania donovani* cells, stationary phase cultures were harvested and immunostained using anti-prohibitin antibody (1:50) at 4°C followed by labelling with gold (18 nm) labelled anti-mouse secondary antibody (1:20). The immunostained cells were fixed (0.8% glutaraldehyde and 4% paraformaldehyde) at 4°C. To confirm the specificity of staining, unrelated antibody was used as a control at similar dilutions. Following wash, the cells were mounted on copper coated grids (TAAB, Berkshire, UK) and whole-cell mounts were viewed using a Jeol 2100F transmission electron microscope (Jeol, Tokyo, Japan) to view surface localization. Anti-Bcl-2 antibody was used as an isotype control antibody in all of the stainings above. Similar protocol was followed for surface immunostaining of macrophage HSP70.

**Treatments**

For cleavage of GPI anchored proteins, cells were treated for 1 h with 0.5 units of PIPLC at 37°C. For neutralization of surface prohibitin of *Leishmania*, 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 h. Undigested antibody was removed from the supernatant with Protein G beads and the Fab fragments were incubated with live parasites at 4°C for 1 h. Fab fragments of anticytosolic tryparedoxin antibody were used to incubate control parasites. Binding efficiency of the parasites was determined by flow cytometry and microscopy as described previously (Iyer et al., 2008).

**Cloning and overexpression of prohibitin**

RNA preparation, cDNA synthesis, PCR amplification and DNA sequencing were carried out as described previously (Iyer et al., 2008) using sense primer 5′-ATG TCG AAG TTG CTG CAG-3′ and antisense primer 5′-TCA CTT CGA CAT GTT CAT CAG-3′ designed based on *L. major* prohibitin sequence (LmjF16.1610, GeneDB of *L. major* database). The prohibitin sequence was aligned with prohibitin sequences of 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. Prosite was used to identify signature motifs (CBS, Technical University of Denmark) while Target P (Emanuelsson et al., 2000) and Phobius (EMBL-EBI) (Kall et al., 2004; Nielsen et al., 1997) were used for prediction of signal sequences. Big PI software (Eisenhaber et al., 2000) was used to predict the possibility of existence of GPI anchors.

**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. Prosite was used to identify signature motifs (CBS, Technical University of Denmark) while Target P (Emanuelsson et al., 2000) and Phobius (EMBL-EBI) (Kall et al., 2004; Nielsen et al., 1997) were used for prediction of signal sequences. Big PI software (Eisenhaber et al., 2000) was used to predict the possibility of existence of GPI anchors.

**Enzyme-linked immunoadsorbant 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 [Tech. Rep. Ser. 793 154 (1990)]. Crude parasite leishmanial antigen (1 μg well-1) or peptide A (RRAAVVR, provisional patent application filed, 474/DEL/2009) (2 μg well-1) was 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 azinobisthiosulfuric acid (ABTS) as the substrate (Chatterjee et al., 1999a). Each point represents the average of four independent experiments.

**Recombinant prohibitin and macrophage membrane reactions**

Recombinant prohibitin was generated using TNT coupled reticulocyte lysate systems (Promega Corporation, Madison, WI, USA). Prohibitin gene encoding T7 promoter-based plasmid (1 μg) was added to the reticulocyte lysate 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 μg/reaction) were added and the mixture was described previously where asparagine 252 was replaced by aspartic acid using the following primers;

(i) N252D Prohibitin sense, 5′-CCAGATCGTGGGCAATGCA GGACGTGACGTTCTGACCGAAAG-3′
(ii) N252D Prohibitin antisense, 5′-TTTCCGTACGAACGTCGCTTGCGATGG-3′

Expression of the fusion protein was checked using the primers described below and the primers used to detect the presence of the GFP–prohibitin fusion protein, GFP protein and actin were:

GFP–Prohibitin sense, 5′-CACTACCAGCAGAACC-3′;
GFP–Prohibitin antisense, 5′-ATTTGGTGCTGACAGAGG-3′;
GFP-sense, 5′-CGCTCTCTCTCTCTCTCT-3′;
GFP-antisense, 5′-GTCCCTCTTGAAGTGCAT-3′;
Actin-sense, 5′-ATGACATGGAGAGATCTGCC-3′
Actin-antisense, 5′-TTCACGTTGCGCACTCTGCC-3′.

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immunoprecipitated with anti-prohibitin antibody as described under ‘Immunoprecipitation and protein microsequencing’, resolved on 12% SDS-PAGE, silver stained, processed for tryptic digestion and microsequencing. For preparation of His-tagged prohibitin sequence, the pET-prohibitin plasmid was transformed into BL21 cells and the expressed protein was purified by routine procedures as described previously (Iyer et al., 2008). The protein was biotinylated using 20 M excess of Sulfo-NHS-biotin; after completion of the reaction the excess biotin was removed using Amicon 10 concentrator by serial dilution and concentration at 4°C.

siRNA transfection

J774A.1 was transfected with 100 pmol HSP70 HP Genome wide siRNA (Qiagen, Hilden, Germany) having a mixture of 4 siRNAs (Access No. NM032327; NM010479; NM031165) using Transpass R2 transfection reagent as per the manufacturer’s protocol. Briefly, HSP siRNA and Cy3 labelled negative siRNA (control siRNA) were added to transfection reagent diluted in serum-free medium and incubated for 20 min to allow the formation of transfection complexes. The siRNA transfection complexes were added at a final concentration of 100 pmol to 10⁵ cells per well grown on 24-well plates and incubated for 6 h followed by addition of fresh complete medium. Transfection efficiency was estimated using flow cytometer by measuring Cy3 fluorescence. Target protein knock-down was assessed 24 h post transfection by surface immunostaining.

Statistical analysis

Data are reported as mean ± SE unless mentioned. Comparisons were made between different treatments using the unpaired Student’s t-test. Differences were considered significant at P < 0.05.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A. The translated protein sequence of L. donovani prohibitin obtained from the DNA sequence (GenBank™ accession number DQ246217). The underlined groups are the peptide sequences obtained from MS/MS analysis of tryptic digests of the immunoprecipitate obtained from Leishmania cell lysates using anti-prohibitin antibody (shown in Fig. 1A, lane c).

B. Figure shows the total profile of peptide fingerprint generated from MS/MS analysis of tryptic digest of prohibitin immunoprecipitate (shown in Fig. 1A, lane c). The sequences share similarity with L. major prohibitin.

Fig. S2. A. Optical sectioning by confocal microscopy of a Leishmania promastigote stained for prohibitin showing localization of prohibitin in the aflagellar pole (AF) and at the flagellar pocket (FP). Kinetoplast (K) and Nucleus (N) is stained with Hoechst 33342. Images a–i each represent 1 μm thick Z-sections of the cell. Scale is 5 μm.

B. Immunostaining of fixed permeabilized cells using anti-prohibitin antibody, a, nomarski image of b–e; b, staining of vesicular structures containing prohibitin (488 nm); c, nuclear and kinetoplast DNA stained with Hoechst 33342 (346 nm); d, mitochondria staining with mitotracker red (578 nm); e, overlap of images from 488 nm, 346 nm and 578 nm illuminations. Scale is 5 μm.

C. Figure shows colocalization studies with fixed and permeabilized cells using ER tracker blue a-d. a, nomarski image; b, staining with ER tracker blue; c, prohibitin staining; d, overlap of b and c. Note no significant overlap. Staining with Lysotracker red shown in e-h. e, nomarski image; f, staining with Lysotracker red; g, prohibitin staining; h, overlap of f and g. Note no significant overlap.

Fig. S3. A. Clustal-W analysis of L. donovani prohibitin with other homologue and orthologues of prohibitin from T. brucei (GenBank™ accession number, XP_824798), T. congoense (congo660n05.p1k_0, GeneDB gene database of T. congoense), T. cruzi (Tc00.1047053511383.50, GeneDB gene database of T. cruzi), L. major (LmjF16.1610, GeneDB gene database of L. major), L. infantum (LinJ16.1700, GeneDB gene database of L. infantum), L. braziliensis (LbrM16.1230, GeneDB gene database of L. braziliensis), P. berghei (PB000121.01.0, GeneDB gene database of P. berghei) and P. falciparum (PF08–0006, GeneDB gene database of P. falciparum), H. sapiens (GenBank™ accession number, AAH95460) and R. norvegicus (GenBank™ accession number, NP_114039). PHB homology domain is marked in blue. Identical amino acids are shown by ‘*’, conserved substitution by ‘:’ and semi-conserved substitution as ‘.’.

Fig. S4. A. Flow cytometric analysis of the HSP70 or secondary antibody stained viable cells showing the mean fluorescence staining of each group. *P < 0.05, secondary control versus HSP70 staining. Data are ± SEM (n = 3).

B. Photomicrographs of immunostained viable J774A.1 macrophages using anti-HSP70 antibody (g) or secondary antibody only (c) showing the presence of surface HSP70 on the macrophage. Nuclei are stained using Hoechst 33342 (b and f), a and e being the respective nomarski images, d and h being the respective merge. Scale is 10 μm.

C. Photomicrographs of immunostained viable murine peritoneal macrophages using anti-HSP70 antibody (g) or secondary antibody only (c) showing the presence of surface HSP70 on the macrophage. Nuclei are stained using Hoechst 33342 (b and f), a and e being the respective nomarski images, d and h being the respective merge. Scale is 10 μm.

Fig. S5. A. Immunoelectron micrograph of macrophages labelled with antiBcl-2 primary antibody and secondary antibody conjugated to gold (18 nm). a–c represent different areas of the cell at higher magnification showing significant localization of the gold particles. Arrows indicate gold particles.

B. Immunoelectron micrograph of macrophages labelled with anti-HSP70 primary antibody and secondary antibody conjugated to gold (18 nm). a–c represent different areas of the cell at higher magnification showing significant localization of the gold particles. Arrows indicate gold particles.

Fig. S6. A. Figure shows colocalization of HSP70 and CD11b; a, nomarski image of J774 macrophages; b, surface staining of live macrophages with anti-HSP70 antibody; c, surface staining of live macrophages with anti-CD 11b antibody; d, merge of the above two stains; e, merge of the above two stains with Hoechst 33342 stain for nuclear stain; f, mask of colocalization of CD-11b and HSP70. Scale is 20 μm.

B. Figure shows colocalization of HSP70 and CD11b; a, nomarski image of murine peritoneal macrophages; b, surface staining of live macrophages with anti-HSP70 antibody; c, surface staining of live macrophages with anti-CD 11b antibody; d, merge of the above two stains; e, merge of the above two stains with Hoechst 33342 stain for nuclear stain; f, mask of colocalization of CD-11b and HSP70. Scale is 20 μm.

Fig. S7. A. Clustal W of L. donovani prohibitin, H. sapiens prohibitin (GenBank™ accession number, AAH95460), M. musculus prohibitin (GenBank™ accession number, NP_032857) and T. cruzi prohibitin (Tc00.1047053511383.50, GeneDB gene database of T. cruzi) showing the presence of a antigenic peptide region in trypanosomatid species, marked in red.

B. Clustal W of L. donovani prohibitin, P. berghei prohibitin (PB000121.01.0, GeneDB gene database of P. berghei) and P. falciparum prohibitin (PF08–0006, GeneDB gene database of P. falciparum) showing the presence of the antigenic peptide region in L. donovani prohibitin.

Fig. S8. Table in the figure shows the clinical laboratory features of sera from VL patients whose sera were used to estimate
reactivity to prohibitin to detect anti-prohibitin antibodies.   

BMI is weight in kilograms divided by height square in meter, normal BMI = 18.5–24.9.  

The Karnofsky performance scale is as follows: 100, able to carry on normal activity and no special care is needed, with no complaints and no evidence of disease; 90, able to carry on normal activity, with minor signs or symptoms of disease; 80, normal activity with effort, with some signs or symptoms of disease; 70, unable to work but able to live at home and care for most personal needs, with various amounts of assistance needed.  

Splenic score is taken as 5, 10–100 parasites/field; 4, 1–10 parasites/field; 3, 1–10 parasites/10 fields; 2, 1–10 parasites/100 fields; 1, 1–10 parasites/1000 fields; 0, no parasites or 1–10 parasites/1000 fields.  

Anti-leishmanial serology was estimated using immobilized crude parasite antigen as described elsewhere (Chatterjee et al., 1999a).  

Increased presence of linkage-specific 9-O-AcSGPs on erythrocytes of active VL was quantified by erythrocyte-binding assay and flow cytometry using FITC-Achatinin-H (Chava et al., 2004).  

Presence of anti-9-O-AcSGP antibodies was determined by ELISA using bovine submandibular mucin (BSM) as the coating antigen as described elsewhere (Chatterjee et al., 1999b).  

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