Leishmania EF-1α Activates the Src Homology 2 Domain Containing Tyrosine Phosphatase SHP-1 Leading to Macrophage Deactivation*

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The human leishmaniasis are persistent infections of macrophages caused by protozoa of the genus Leishmania. The chronic nature of these infections is in part related to induction of macrophage deactivation, linked to activation of the Src homology 2 domain containing tyrosine phosphatase-1 (SHP-1) in infected cells. To investigate the mechanism of SHP-1 activation, lysates of Leishmania donovani promastigotes were subjected to SHP-1 affinity chromatography and proteins bound to the matrix were sequenced by mass spectrometry. This resulted in the identification of Leishmania elongation factor-1α (EF-1α) as a SHP-1-binding protein. Purified Leishmania EF-1α, but not host cell EF-1α, bound directly to SHP-1 in vitro leading to its activation. Three independent lines of evidence indicated that Leishmania EF-1α may be exported from the phagosome thereby enabling targeting of host SHP-1. First, cytosolic fractions prepared from macrophages infected with [35S]methionine-labeled organisms contained Leishmania EF-1α. Second, confocal, fluorescence microscopy using Leishmania-specific antisera detected Leishmania EF-1α in the cytosol of infected cells. Third, co-immunoprecipitation showed that Leishmania EF-1α was associated with SHP-1 in vivo in infected cells. Finally, introduction of purified Leishmania EF-1α, but not the corresponding host protein into macrophages activated SHP-1 and blocked the induction of inducible nitric-oxide synthase expression in response to interferon-γ. Thus, Leishmania EF-1α is identified as a novel SHP-1-binding and activating protein that recapitulates the deactivated phenotype of infected macrophages.

According to the latest WHO report, 12 million people are affected by leishmaniasis worldwide and 2 million new cases occur each year (Leishmaniases Control, www.who.int/health-topics/leishmaniasis.htm, updated 2000). Moreover, the incidence of the leishmaniasis has been on the rise because of multiple factors including the AIDS epidemic, increased international travel, lack of effective vaccines, difficulty in controlling vectors, international conflicts, and the development of resistance to chemotherapy. Progress in controlling the leishmaniasis will require improved understanding of pathogenesis to identify novel drug targets or vaccine candidates.

Leishmania donovani is the major causative agent of human visceral leishmaniasis. Leishmania live as either extracellular, flagellated promastigotes in the digestive tracts of their sand fly vectors or as nonflagellated amastigotes within macrophages, where they survive and replicate within phagolysosomes. Macrophages as part of both the innate and acquired immune systems are programmed to ingest and destroy intracellular pathogens. Hence, the mechanisms used by Leishmania and other intracellular pathogens to evade elimination by macrophages are important issues in cell biology and immunology. Infected macrophages are often refractory to cell activation (1, 2) and recent evidence suggests that this is related to impaired cell signaling (1–4) brought about by the action of protein-tyrosine phosphatase (5). In particular, several lines of evidence have converged to establish a role for the host macrophage Src homology 2 domain containing protein tyrosine phosphatase-1 (SHP-1) in the pathogenesis of infection with Leishmania (4, 6–9). Notably, infected macrophages show increased SHP-1 activity and inhibition of tyrosine phosphatase activity reverses the abnormal phenotype of infected cells (4). Moreover, resistance to Leishmania infection is enhanced in SHP-1-deficient macrophages and mice (8). The argument that activation of host SHP-1 is involved in the pathogenesis of infection with Leishmania is also supported by the recent finding that the first-line anti-leishmanial agent used clinically, sodium stibogluconate, is an inhibitor of SHP-1 (10).

Although much is known about the mechanisms that regulate SHP-1 activation, few discrete SHP-1-activating ligands have been identified (11, 12). Here, we show that elongation factor-1α (EF-1α) of L. donovani is a novel SHP-1-binding protein and SHP-1 activator, properties not shared by the corresponding host protein. The results also show that introduction of Leishmania EF-1α, but not host EF-1α into cells, recapitulates the deactivated phenotype of Leishmania-infected macrophages. These findings identify Leishmania EF-1α as a novel, candidate virulence factor.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—RPMI 1640, Hanks’ balanced salt solution, and protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, pep-

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1 The abbreviations used are: SHP-1, Src homology 2 domain containing tyrosine phosphatase; EF-1α, elongation factor-1α; INOS, inducible nitric-oxide synthase; GST, glutathione S-transferase; pNPP, p-nitrophenyl phosphate; IFN-γ, interferon-γ; ITIM, immunoreceptor tyrosine-based inhibitory motif.
Leishmania EF-1α Activates SHP-1

50191

stain A, and leupeptin), 1.4-diazabicyclo[2.2.2]octane, DEAE-Sepharose, CM-Sepharose CL-6B, cellulose phosphate (fibrous form), anti-mouse IgG-fluorescein, and calmodulin-agarose were obtained from Sigma. Medium 199 was from Invitrogen. The RAW 264.7 cell line was from the American Type Culture Collection (Rockville, MD). Anti-EF-1α was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-α-Tubulin and anti-cathepsin D were from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Horseradish peroxidase-conjugated goat anti-rabbit antibodies, protein G-agarose, and electrophoresis reagents and supplies were from Bio-Rad. Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences. Preparation of the GST-SHP-1 construct, its expression and purification has previously been described (13). Protein delivery system Profect 1 was obtained from Targeting Systems San Carlos, CA.

L. donovani—Amastigotes of the Sudan strain 2S of L. donovani were maintained by serial intracardiac inoculation of amastigotes into female Syrian hamsters. Amastigotes were isolated from the spleens of infected hamsters by culturing in medium 199 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), adenosine (1 mM), folic acid (10 μg/ml), and hemin (6 μg/ml) at 37 °C for 20 min. After incubation with antibodies, protein G-agarose, and electrophoresis reagents and supplies were from Bio-Rad. Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences. Preparation of the GST-SHP-1 construct, its expression and purification has previously been described (13). Protein delivery system Profect 1 was obtained from Targeting Systems San Carlos, CA.

Infection of RAW Cells with L. donovani—Exponentially growing RAW cells were infected with either stationary phase promastigotes or freshly isolated amastigotes at a parasite to cell ratio of ~10:1. After incubation at 37 °C for 5% CO₂, noninternalized parasites were removed by washing with Hanks' balanced salt solution. To determine the rate of infection, cytosin preparations were prepared from dislodged cells, which were stained with Diff-Quik.

Cell Culture—The murine macrophage cell line RAW 264.7 was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere (5% CO₂).

Infection of RAW Cells with L. donovani—Exponentially growing RAW cells were infected with either stationary phase promastigotes or freshly isolated amastigotes at a parasite to cell ratio of ~10:1. After incubation at 37 °C for 5% CO₂, noninternalized parasites were removed by washing with Hanks' balanced salt solution. To determine the rate of infection, cytosin preparations were prepared from dislodged cells, which were stained with Diff-Quik.

Cytocentrifugation—Leishmania promastigotes (2–3 × 10⁶) were lysed in ice-cold lysis buffer A (50 mM Tris, pH 7.5, 0.5% Triton X-100, and 20 mM NaCl) containing a mixture of protease inhibitors for 20 min on ice. All subsequent steps were performed at 4 °C. Lysates were centrifuged in a microcentrifuge at maximum speed for 10 min and supernatants were incubated with GST-SHP-1 affinity beads with end-over-end rotation for 2 h. After incubation, affinity beads were transferred to a column and washed extensively. Bound proteins were released with buffer A containing 0.5 M NaCl. An aliquot of partially purified SHP-1 bound proteins was subjected to SDS-PAGE (12%) followed by silver staining.

Immunoprecipitation and Immunoblotting—L. donovani promastigote and RAW cell lysates were subjected to centrifugation and washed twice with Tris-buffered saline, pH 7.4, and immediately processed for immunoprecipitation. For immunoprecipitation of EF-1α, parasites were lysed on ice in lysis buffer B (50 mM Tris, pH 7.4, 1% Triton X-100, 0.15 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin/ml, and 2 μg/mL of pepstatin A/mL). Cell lysates were clarified by centrifugation in a microcentrifuge at maximum speed for 20 min at 4 °C. The resulting supernatants were incubated with anti-EF-1α antibodies for 16 to 18 h at 4 °C. After incubation with antibodies, protein G-agarose was added for 2 h at 4 °C for recovery of immune complexes. After extensive washing, immune complexes were released by boiling 10 min in SDS-PAGE sample buffer with β-mercaptoethanol. The samples were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system. The membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline followed by incubation with anti-EF-1α antibodies. After washing, the blots were incubated with anti-mouse horseradish peroxidase-conjugated antibody using the ECL chemiluminescence system.
Identification of Leishmania EF-1α as a SHP-1-binding Protein—Initial studies showed that crude lysates from both Leishmania promastigotes and amastigotes contained a factor that activated SHP-1 in vitro (data not shown). To investigate this further, affinity chromatography of Leishmania lysates was carried out using GST-SHP-1 coupled to glutathione-Sepharose as matrix. Two prominent proteins with approximate subunit sizes of 56 and 44 kDa specifically bound to GST-SHP-1 (Fig. 1A). A parallel affinity column consisting of GST-glutathione-Sepharose showed no detectable binding proteins (data not shown). The two bands shown in Fig. 1A were excised from the gel, and tryptic peptide digests were analyzed by mass spectrometry. Whereas the identity of the 44-kDa band is as yet undetermined, eight of the peptides (TATGLHYK, TIEKFEK, YAWVLDKL, VGYNVEK, SENMPWYK, LPLQDVYK, IG-GIGTVPGV, and KFAIEIESK) sequenced from the 56-kDa silver-stained band were found to match EF-1α from Leishmania braziliensis (NCBI nr accession number 5834626) and covered 16.2% of the sequence.

To confirm the identity of the 56-kDa protein, GST-SHP-1-binding proteins were prepared from a Triton X-100 lysate of stationary phase L. donovani (Ld) promastigotes. The original Leishmania lysate and the proteins eluted from the column were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-EF-1α antibody. Single bands of identical size in both the Leishmania lysate and in the GST-SHP-1 affinity column eluate (lane 1 and 2 in Fig. 1B) were detected, thus confirming the internal protein sequence data indicating that the 56-kDa SHP-1-binding protein band contained EF-1α. Notably, Leishmania EF-1α was observed to be larger than its human homologue (compare lanes 1 and 2 with lane 3 in Fig. 1B). These results identified Leishmania EF-1α as a SHP-1-binding protein.

Leishmania EF-1α and Host EF-1α Show Differential Binding to SHP-1 in Vitro—To investigate further the interaction of Leishmania EF-1α with SHP-1, EF-1α was purified to near homogeneity from murine macrophages and from Leishmania promastigotes as described under “Experimental Procedures.” The fractions containing homogeneous EF-1α as judged by immunoblotting (data not shown) and silver staining (Fig. 2A and B) were pooled and dialyzed. The purity of these preparations was examined further by tryptic digestion and analysis by mass spectrometry and showed no detectable contamination by other proteins (data not shown). Purified EF-1α from both sources was incubated with GST-SHP-1 and binding assays were performed as described under “Experimental Procedures.” The results in Fig. 3A show that EF-1α from Leishmania bound directly and selectively to SHP-1 as comparatively little binding of host EF-1α was detected. To determine that the relatively poor binding of host EF-1α to SHP-1 was not related to loss of functional integrity, both preparations of purified EF-1α were used in binding assays with calmodulin-agarose beads. These findings indicated that purified host EF-1α was not bound to SHP-1, nor was it able to activate SHP-1 in vitro. Purified EF-1α from Leishmania, macrophages, or an equivalent amount of bovine serum albumin
binding assays performed in duplicate. One of two independent experiments that yielded similar results.

After overnight infection, cells were disrupted and cytosolic fractions were isolated as described under "Experimental Procedures." For binding to SHP-1 (A), purified EF-1α from either Leishmania or macrophages was incubated with 25 μl (packed volume) of glutathione-agarose beads containing equal amounts of GST-SHP-1. Binding was accomplished by mixing in 100 μl of binding buffer for 2 h at 4°C. The beads were collected by centrifugation and washed four times with binding buffer. Bound EF-1α was eluted by boiling beads in Laemmli sample buffer, and separated on SDS-PAGE followed by immunoblotting using anti-EF-1α. Lanes 1 and 5 represent input amounts of purified EF-1α, lanes 2, 3, 6, and 7 are binding assays performed in duplicate. Binding of EF-1α to calmodulin-agarose was performed essentially as described above for GST-SHP-1 except that binding buffer contained 0.2 mM CaCl2 instead of EDTA. Lanes 1 and 4 represent input amounts of purified EF-1α, lanes 2, 3, 5, and 6 are duplicate binding assays. The results shown are from one of two independent experiments that yielded similar results.

min were mixed separately with Profect 1 reagent in serum-free medium for delivery to macrophages. After 2–3 h of incubation, cells were lysed and immunoprecipitated for SHP-1. Immune complexes were recovered using protein A-Sepharose and after extensive washing, immune complexes were assayed for phosphatase activity using pNPP as a substrate. Relative phosphatase activity was assessed by measuring changes in absorbance at 410 nm. B, in vitro activation of macrophage SHP-1. Approximately 1 μg of purified EF-1α prepared from either Leishmania or macrophages or an equivalent amount of bovine serum albumin was incubated separately with Profect 1 reagent in serum-free media to prepare protein-Profect 1 complexes for delivery to RAW264.7 cells (2 × 10^6) according to the manufacturer's instructions. After 2–3 h of incubation, cells were lysed in buffer C containing a mixture of protease inhibitors for 20 min on ice and incubated with anti-SHP-1 for 2 h at 4°C. Immune complexes were recovered using protein A-Sepharose and after extensive washing with buffer C, immune complexes were assayed for phosphatase activity using pNPP as a substrate. The results shown are from one of three independent experiments that yielded similar results.

Leishmania EF-1α Accesses the Cytosol of Infected Macrophages—SHP-1 is predominantly a cytosolic protein, whereas Leishmania reside within a membrane-bound vacuole. This raises an important question of whether a factor from vacuole-bound Leishmania could access cytosolic SHP-1. To examine the subcellular distribution of Leishmania EF-1α in infected macrophages, Leishmania promastigotes were biosynthetically labeled with [35S]methionine, washed to remove unincorporated isotope, and immediately used to infect macrophages. After overnight infection, cells were disrupted and cytosolic fractions were isolated as described under "Experimental Procedures." EF-1α was immunoprecipitated from the cytosolic, infected cells under native conditions. Immunoprecipitated proteins were separated by SDS-PAGE and detected by autoradiography. Anti-EF-1α precipitated a protein of the correct size for Leishmania EF-1α (Fig. 5A, lane 2) suggesting the presence of Leishmania EF-1α in cytosol of infected macrophages. No [35S]methionine-labeled proteins were precipitated using irrelevant antibody (Fig. 5A, lane 1). To control for the possibility that disruption of phagolysosomes during cell frac-

tionation resulted in the release of EF-1α, cytosolic fractions were examined for the presence of mature cathepsin D, a marker of phagolysosomes. The absence of detectable cathepsin D in the cytosol of infected cells (Fig. 5B, lane 1) suggests that any disruption of phagolysosomes was negligible and thus did not account for the presence of Leishmania EF-1α this fraction.

The presence of Leishmania EF-1α in cytosol of infected macrophages was also investigated using polyclonal antibody raised against Leishmania EF-1α peptide. The specificity of this antibody for Leishmania EF-1α was established by immunoblotting using total cell lysates of Leishmania and macrophages. The presence of a single band in the region of Leishmania EF-1α in lysates of Leishmania and no detectable band in lysates of macrophages (Fig. 5C, compare lanes 1 and 2) clearly shows that this antisera is highly specific for Leishmania EF-1α. The specificity of anti-Leishmania EF-1α was confirmed using purified EF-1α from Leishmania and macrophages (data not shown). When the cytosolic fraction from infected macrophages was subjected to immunoblotting with this anti-peptide antisera, the results showed (Fig. 5D) the presence of Leishmania EF-1α in this fraction.

Direct Localization of Leishmania EF-1α in Infected Macrophages—Scanning, confocal, immunofluorescence microscopy using antipeptide antisera was used to independently confirm the presence of Leishmania EF-1α in the cytosol of infected macrophages. Infected and control macrophages grown on coverslips were fixed, permeabilized, and processed for immunofluorescence using either anti-peptide antisera specific for Leishmania EF-1α or commercial anti-EF-1α antisera that recognizes both Leishmania and mammalian EF-1α. Using Leishmania-specific anti-peptide antisera, the results shown in Fig. 6, panels 2 and 3, demonstrate Leishmania EF-1α in the cytosol of infected macrophages. As expected, staining of control, noninfected macrophages using the Leishmania-specific antisera showed no signal (Fig. 6, panel 5), however, control
cells showed intense, diffuse fluorescence when commercial anti-EF-1α antibody was used for staining (Fig. 6, panel 7). Taken together with the immunoprecipitation and Western blotting findings, direct detection using confocal microscopy, provides compelling evidence indicating the presence of Leishmania EF-1α in the cytosolic fraction of infected macrophages.

Leishmania EF-1α Associates with Host SHP-1 in Vivo—The findings that Leishmania EF-1α bound to SHP-1 in vitro and evidence that it accessed the cytosol of infected cells, suggested that it may target SHP-1 in vivo. To examine this possibility, macrophages were infected with promastigotes for 14–16 h. Cytosolic fractions were then prepared from control and infected cells for immunoprecipitation of SHP-1. Immune complexes were separated on SDS-PAGE under nonreducing conditions followed by transfer to nitrocellulose. Immunoblot analysis carried out using anti-EF-1α (Fig. 7, lane 4) showed that Leishmania EF-1α was associated with SHP-1 in vivo, whereas the association of host-EF-1α with host SHP-1 was not detectable.

Leishmania EF-1α Attenuates IFN-γ-induced Activation of iNOS Expression in Macrophages—In parallel with its ability to activate SHP-1, we examined whether the introduction of EF-1α into cells would impact macrophage activation. Purified Leishmania or macrophage EF-1α was introduced into macrophages using Profect 1 reagent. After incubation for 2 h, macrophages were stimulated with murine IFN-γ. Cells were then lysed, separated on SDS-PAGE, transferred to nitrocellulose, and probed with anti-iNOS. As shown in Fig. 8, the delivery of purified, native Leishmania EF-1α, but not the corresponding host protein into cells blocked the induction of iNOS expression in response to cell treatment with interferon-γ. The same blot was stripped and reprobed with anti-actin to control for protein loading. Thus, Leishmania EF-1α was able to recapitulate the deactivated phenotype of Leishmania-infected macrophages.

**DISCUSSION**

Results from several recent studies have suggested that the protein-tyrosine phosphatase SHP-1 plays a role in the pathogenesis of Leishmania infection (4, 6, 7). Based upon the particular finding that infection of macrophages with Leishmania results in SHP-1 activation (4, 7), we sought to determine whether a Leishmania activator of SHP-1 could be found. To identify potential SHP-1 interacting protein(s) from Leishmania, affinity chromatography of Leishmania lysates was carried out using GST-SHP-1 coupled to glutathione-Sepharose. This resulted in the isolation of a 56-kDa SHP-1-binding protein that was identified as EF-1α (Fig. 1).

The finding that SHP-1 and Leishmania EF-1α were binding partners was unexpected because EF-1α is a highly conserved, ubiquitously expressed protein in all eukaryotic cells with a diverse range of regulatory properties (15, 17–19). Certainly the role that EF-1α is best known for, if not its most important one, is as a GTP-binding protein involved in regulating the rate and fidelity of protein translation (17, 18). EF-1α exists as a multimeric complex in which the α subunit binds to both GTP and aminoacyl-tRNA, whereas the β and γ subunits are in-

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**Fig. 5. Detection of Leishmania EF-1α in the cytosol of infected macrophages.** A, autoradiograph of immunoprecipitated EF-1α. [35S]Methionine-labeled promastigotes were used to infect ~4 × 10⁶ macrophages at a parasite to cell ratio of 10:1. After incubation for 16 h, cells were processed to isolate a cytosolic fraction for immunoprecipitation using anti-EF-1α or isotype-matched irrelevant antibody. Immunoprecipitated proteins were separated by SDS-PAGE and detected by autoradiography. Lane 1, irrelevant antibody; lane 2, anti-EF-1α. B, immunoblotting to detect cathepsin D in the cytosolic fraction of infected macrophages. Approximately 100 μg of proteins from the cytosolic or pellet (containing phagosomes) fractions from infected macrophages were precipitated using ice-cold trichloroacetic acid (10% final). Trichloroacetic acid precipitates were washed with cold acetone, air dried, solubilized in sample buffer, and separated by SDS-PAGE followed by transfer to nitrocellulose. The membranes were probed with anti-cathepsin D antibodies. Lane 1, cytosolic proteins; lane 2, proteins from the pellet fraction. C, specificity of Leishmania EF-1α anti-peptide antibody. Approximately 50 μg of Triton X-100-soluble extracts from RAW264.7 cells or Leishmania promastigotes were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were probed either with Leishmania EF-1α anti-peptide antibodies or commercial anti-EF-1α. Lane 1, Leishmania cell lysate probed with anti-Leishmania EF-1α anti-peptide antibodies; lane 2, macrophage cell lysate probed with anti-Leishmania EF-1α anti-peptide antibodies; lane 3, macrophage cell lysate probed with commercial anti-EF-1α antibodies. D, approximately 100 μg of cytosolic proteins from infected macrophages were processed for immunoblotting as described above and probed with Leishmania EF-1α-specific anti-peptide antibodies. The results shown are from one of three independent experiments that yielded similar results.

**Fig. 6. Subcellular localization of Leishmania EF-1α in infected macrophages using confocal, immunofluorescence microscopy.** Macrophages were grown on coverslips and either untreated or infected with L. donovani promastigotes for 16 h. Monolayers were then washed and control (panels 4–7) and infected (panels 1–3) macrophages were fixed with paraformaldehyde, permeabilized using Triton X-100, and stained with either Leishmania EF-1α-specific antibodies (panels 2 and 5) or anti-EF-1α commercial antibodies (panel 7). Panels 1, 4 (infected macrophages), and 6 (noninfected) represent differential interference contrast images. Panel 3 represents superimposition of the images in panels 1 and 2. Arrows in panels 1–3 mark the localization of Leishmania in infected cells. The results shown are from one of two independent experiments that yielded similar results.
EF-1α is a phagosome-associated protein also suggests a potential regulatory role in phagocytosis (26).

Given that EF-1α is both highly conserved and ubiquitously expressed, it was important to determine whether host EF-1α also interacted with SHP-1. Of note was the finding that Leishmania EF-1α migrated more slowly than its human homologue during SDS-PAGE (Fig. 1B), suggesting the possibilities of structural and functional differences. To examine this question further, L. donovani EF-1α and macrophage EF-1α were purified by electrophoresis homogeneity (Fig. 2) and used to investigate possible direct interactions with GST-SHP-1. The results shown in Fig. 3A clearly show that SHP-1 binds preferentially to EF-1α from Leishmania, as very little binding of macrophage EF-1α was observed. It has previously been shown that EF-1α of Trypanosoma brucei and mammalian origins directly interacts with calmodulin and this interaction seems to require EF-1α in its native conformation (15). Hence, as a positive control, binding assays were performed using purified EF-1α and calmodulin-agarose beads. The findings that both macrophage and Leishmania EF-1α bound to calmodulin (Fig. 3B) clearly showed that the purification scheme did not disturb the functional integrity of macrophage EF-1α. Taken together, the data show that SHP-1 interacts selectively with Leishmania EF-1α and suggest important structural and functional differences between these otherwise highly homologous proteins.

The specificity of binding of the phosphatase to Leishmania EF-1α suggested that the latter may be a unique SHP-1 regulator. Indeed, just as the Leishmania protein interacted specifically with SHP-1, we found that it functioned as a SHP-1-activating protein both in vitro (Fig. 4A) and in vivo (Fig. 4B), whereas macrophage EF-1α had no such activities. The findings discussed thus far suggested a potential model in which Leishmania EF-1α functions as a virulence factor by activating SHP-1 leading to macrophage deactivation. The requirement in this model for an interaction between these two proteins had to be reconciled with the fact that SHP-1 is predominantly cytosolic in distribution whereas Leishmania reside within a membrane-bound vacuole. The question of whether Leishmania EF-1α could access the macrophage cytosol was addressed using three independent approaches and the evidence from each indicated the presence of Leishmania EF-1α in the cytosol of infected cells. These approaches included: 1) infection of macrophages with 35S-labeled Leishmania followed by immunoprecipitation of labeled EF-1α from cytosolic fractions (Fig. 5A); 2) immunoblotting of the cytosol from infected cells using anti-peptide antiserum specific for Leishmania EF-1α (Fig. 5D); and 3) direct detection of Leishmania EF-1α in the cytosol of infected macrophages using Leishmania EF-1α-specific antiserum combined with confocal microscopy (Fig. 6).

These findings indicate that Leishmania EF-1α was exported from the phagosome where it could target host cell proteins. To examine whether Leishmania EF-1α interacted with host SHP-1 in vivo, SHP-1 was immunoprecipitated from cytosol of Leishmania-infected macrophages followed by immunoblotting using anti-EF-1α. The results (Fig. 7B) clearly showed that Leishmania EF-1α associated with SHP-1 in vivo, whereas the association of host EF-1α with host SHP-1 was not detectable. It is known that the mature form of cathepsin D is present in the lumen of L. donovani containing phagosomes (27, 28). Hence, cathepsin D was used as a marker of phagosome disruption that may have occurred during preparation of cytosolic fractions from infected macrophages. This analysis showed that the subcellular fractionation did not lead to phagosome disruption as no cathepsin D was detected in the cytosol of infected cells even after prolonged exposure of membranes to film (Fig. 5B).
To investigate the importance of the interaction of Leishmania EF-1α with SHP-1, we examined whether the introduction of EF-1α into cells would impact cell activation. The results of this analysis showed that introduction of purified, native Leishmania EF-1α, but not the corresponding host protein into cells blocked the induction of iNOS expression in response to cell treatment with interferon-γ (Fig. 8). Thus, Leishmania EF-1α was able to recapitulate the deactivated phenotype of Leishmania-infected macrophages (4). Taken together, these results show that Leishmania EF-1α is a novel SHP-1 regulator capable of inducing macrophage deactivation. In light of the findings that activation of host SHP-1 appears to be associated with progressive leishmaniasis (4, 6–8) these results strongly suggest that Leishmania EF-1α contributes to disease pathogenesis.

The identification of Leishmania EF-1α as a regulator of host SHP-1 suggests that it may be a novel virulence factor. It also suggests a new paradigm for chronic intracellular infection in which microbial factors directly modulate the activity of host cell regulatory proteins leading to cell deactivation. Clearly, the findings that vanadate treatment reverses the deactivated phenotype of Leishmania-infected macrophages (4) and that SHP-1-deficient cells and mice show enhanced resistance to Leishmania infection (8) are consistent with such a mechanism. A model such as this raises at least two important questions. First, how does EF-1α from vacuole-bound Leishmania cross two membranes (the parasite plasma membrane and the parasitophorous vacuole) to access cytosolic SHP-1? Second, what is the mechanism of phosphatase activation?

Observations addressing the export of macromolecules from Leishmania phagosomes are limited. However, because Leishmania and other intracellular pathogens that reside within phagosomes must access the cytosol to acquire essential nutrients, it seems highly likely that there must be mechanisms by which pathogen-derived molecules can travel in the opposite direction. In this regard, recent work suggests that numerous Mycobacterium bovis BCG proteins are released from phagosomes and can be found in various intracellular compartments (29). Leishmania are known to export a large range of proteins and other factors. For example, both amastigotes and promastigotes secrete a complex range of glycoconjugates including lipophosphoglycan that are surface expressed and secreted (30). Leishmania also export a range of proteophosphoglycans important for interactions within the sandy gut and for virulence in mammals. Several of these factors are released into phagosomes (31–35) and at least two of these have also been detected in parasite-free vesicles within macrophages (31, 34). Leishmania use a conventional eukaryotic secretory pathway to translocate proteins with signal sequences into the endoplasmic reticulum (36). However, proteins exported by Leishmania that lack a signal sequence have also been identified such as the family of hydrophilic acylated surface proteins. Recently, an acylation-dependent process of protein export in Leishmania involving both palmitoylation and myristoylation has been described for the hydrophilic acylated surface protein (37). It has recently been shown that Leishmania phagosomes can access macromolecules from the macrophage cytosol (38) and Leishmania promastigotes have been described that act to create permeable phagosomes (39). In regard to export of EF-1α from the phagosome, this is unlikely to involve a classical secretory mechanism because the predicted amino acid sequence of EF-1α shows no classical signal sequence for secretion. Hence, this likely involves a nonclassical secretory pathway, perhaps acylation-dependent or otherwise.

As to the mechanism by which Leishmania EF-1α activates SHP-1, it is known that SHP-1 activity is increased as a result of the binding of its tandem Src homology 2 domains to tyrosine-phosphorylated, immunoreceptor tyrosine-based inhibitory motifs (ITIM) within regulatory proteins (11, 12). Our findings that Leishmania EF-1α is tyrosine phosphorylated and that the sequence contains two canonical ITIM motifs is consistent with such a model. This mechanism has to be reconciled, however, with the fact that these ITIMs are conserved within mammalian EF-1α, which does not activate SHP-1 (Fig. 4). Structural differences between these homologues may account for these divergent activities. For example, in addition to a difference in molecular size (Fig. 1), alignment of mammalian and the EF-1α of Leishmania revealed several significant structural differences that could conceivably make these ITIMs or other motifs unavailable in the host protein. This analysis showed that despite its slower migration during SDS-PAGE the Leishmania protein has a shorter amino acid sequence compared with mammalian EF-1α by virtue of a 12-amino acid deletion. Comparison of the sequences showed several other critical amino acid substitutions that could contribute to structural and functional differences.

Other than activation by ITIMs, the activity of SHP-1 may also be influenced by its phosphorylation on tyrosine (40, 41) and by interactions with phospholipids (42, 43). Thus, an additional possibility to consider is that the binding of Leishmania EF-1α to SHP-1 may influence the subcellular distribution of the phosphatase and its proximity to a microdomain such as a lipid raft enriched in an activating tyrosine kinases or phospholipid.

In summary, this study has demonstrated a selective interaction between SHP-1 and Leishmania EF-1α and has identified the latter as a novel SHP-1 regulator. By virtue of the fact that this interaction leads to macrophage deactivation, Leishmania EF-1α is identified as a candidate virulence factor. Evidence that Leishmania EF-1α may be a novel virulence determinant combined with its significant structural and functional differences when compared with the corresponding host protein, suggest that it may be an attractive target for anti-Leishmania drug or vaccine development. This potential may be even greater given the essential requirement for EF-1α in protein synthesis and other critical cell functions.

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