Microbiology:

The Plasma Membrane of Bloodstream-form African Trypanosomes Confers Susceptibility and Specificity to Killing by Hydrophobic Peptides

John M. Harrington, Justin Widener, Natalie Stephens, Thomas Johnson, Maria Francia, Paul Capewell, Annette Macleod and Stephen L. Hajduk

J. Biol. Chem. 2010, 285:28659-28666.
doi: 10.1074/jbc.M110.151886 originally published online July 8, 2010

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 37, pp. 28659–28666, September 10, 2010

The Plasma Membrane of Bloodstream-form African Trypanosomes Confers Susceptibility and Specificity to Killing by Hydrophobic Peptides*[S]

Received for publication, June 4, 2010, and in revised form, July 7, 2010 Published, JBC Papers in Press, July 13, 2010, DOI 10.1074/jbc.M110.151886

John M. Harrington[‡1], Justin Widener[‡], Natalie Stephens[‡], Thomas Johnson[‡], Maria Francia[‡], Paul Capewell[§2], Annette Macleod[§3], and Stephen L. Hajduk[‡4]

From the [‡]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602 and the [§]Wellcome Trust Centre for Molecular Parasitology, Glasgow Biomedical Research Centre, Faculty of Veterinary Medicine, University of Glasgow, Glasgow G12 8TA, Scotland, United Kingdom

***Trypanosoma brucei* is the causative agent of both a veterinary wasting disease and human African trypanosomiasis, or sleeping sickness. The cell membrane of the developmental stage found within the mammalian host, the bloodstream form (BSF), is highly dynamic, exhibiting rapid rates of endocytosis and lateral flow of glycosylphosphatidylinositol-anchored proteins. Here, we show that the cell membrane of these organisms is a target for killing by small hydrophobic peptides that increase the rigidity of lipid bilayers. Specifically, we have derived trypanocidal peptides that are based upon the hydrophobic N-terminal signal sequences of human apolipoproteins. These peptides selectively partitioned into the plasma membrane of BSF trypanosomes, resulting in an increase in the rigidity of the bilayer, dramatic changes in cell motility, and subsequent cell death. No killing of the developmental stage found within the insect midgut, the procyclic form, was observed. Additionally, the peptides exhibited no toxicity toward mammalian cell lines and did not induce hemolysis. Studies with model liposomes indicated that bilayer fluidity dictates the susceptibility of membranes to manipulation by hydrophobic peptides. We suggest that the composition of the BSF trypanosome cell membrane confers a high degree of fluidity and unique susceptibility to killing by hydrophobic peptides and is therefore a target for the development of trypanocidal drugs.**

The eukaryotic parasite *Trypanosoma brucei* infects mammals, causing a wasting disease in cattle and a disease in humans known as African trypanosomiasis, or sleeping sickness. The human pathogens are two subspecies of *T. brucei*, *T. brucei rhodesiense* and *T. brucei gambiense*, causing acute and chronic infections, respectively (1). The subspecies *T. brucei brucei* does not infect humans or other higher primates due to its susceptibility to the trypanolytic activity of a minor subset of high-density lipoproteins (2, 3).

The developmental stage of *T. brucei* found within the mammalian host, the bloodstream form (BSF),[5] is a highly motile cell (4, 5) and exhibits rapid rates of endocytosis (6) and free diffusion of the densely packed glycosylphosphatidylinositol-anchored variant surface glycoproteins (VSGs) (7). Endocytosis is restricted to the flagellar pocket, a small specialized membrane structure at the posterior of the cell (8). This morphology requires that surface-associated proteins destined for endocytosis be laterally sorted in the plane of the membrane to the flagellar pocket. Perhaps the most striking example of this activity is the sorting of immunoglobulin-bound VSGs to the flagellar pocket by physical forces generated from the flow of extracellular medium over the surface of the trypanosome (9). The composition of the trypanosome plasma membrane that facilitates rapid lateral sorting is not known. In the case of VSGs, surface flow may benefit from the use of myristate, a relatively short acyl chain, as the membrane-anchoring moiety (10, 11). The procyclic form (PCF) of *T. brucei*, found within the insect midgut, does not exhibit rapid rates of endocytosis (12, 13), and the glycosylphosphatidylinositol-anchored procyclin surface proteins are anchored with longer palmitoyl- and stearoyl-acyl chains (14).

The structure of the plasma membrane is also important for avoiding lysis by host defense factors such as complement or antimicrobial peptides. Human defensins, antimicrobial peptides with a distinct tertiary structure, are relatively inefficient at killing BSF *T. brucei* (15), and it may be the case that the VSG coat inhibits interaction with the cell membrane. Interestingly, BSF and PCF *T. brucei* exhibit differential susceptibility to a number of antimicrobial peptides (15). This distinction might be attributed to the different surface density of glycosylphosphatidylinositol-anchored proteins between the two forms, with VSGs being present at roughly an order of magnitude greater than the procyclin coat proteins, thereby providing greater steric hindrance. Alternatively, or in addition to the difference in surface protein density, the differences in phos-

* This work was supported, in whole or in part, by National Institutes of Health Grant AI039033 (to S. L. H.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Movies 1–4.

[1] Supported by a National Research Service Award from NIAID.

[2] United Kingdom Biotechnology and Biological Sciences Research Council research student.

[3] Wellcome Trust Research Career Development Fellow.

[4] To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Georgia, B129 Life Sciences Bldg., 120 Green St., Athens, GA 30602. Tel.: 706-542-1676; Fax: 706-542-0182; E-mail: shajduk@bmb.uga.edu.

[5] The abbreviations used are: BSF, bloodstream form; VSG, variant surface glycoprotein; PCF, procyclic form; SHP, small hydrophobic peptide; PC, phosphatidylcholine; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; DIC, differential interference contrast.

pholipid and sterol composition between the two developmental forms may play a role.

In this study, we describe the trypanocidal activity of small hydrophobic peptides (SHPs), derived from the signal sequences of the human apolipoproteins haptoglobin-related protein (SHP-1) and paraoxonase-1 (SHP-2) (16, 17). These peptides are unusual in that they are retained in the mature secreted protein, having avoided cleavage in the endoplasmic reticulum. SHP-1 circumvents steric hindrance from the VSG coat and interacts with the plasma membrane of BSF *T. brucei*. We show that this non-cationic peptide specifically targets fluid membranes. SHP-1 rapidly binds BSF cells rather than PCF or mammalian cells. Consistently, BSF *T. brucei* cells are readily killed by SHP-1, and no lysis or killing is observed with PCF *T. brucei*, mammalian cell lines, or human erythrocytes. We suggest that the same composition that facilitates surface membrane dynamics in BSF *T. brucei* renders the parasite susceptible to killing by hydrophobic peptides and that the plasma membrane represents a target for the development of trypanocidal agents.

EXPERIMENTAL PROCEDURES

Peptides—Synthetic peptides corresponding to the N-terminal signal peptides of the human apolipoproteins haptoglobin-related protein (SDLGAVISLLLWGRQLFA) and paraoxonase-1 (AKLIATLLGMGLALFRNHQS) and all derivatives were purchased from Bio-Synthesis, Inc. (Lewisville, TX). A hydrophilic peptide (ERTEESWGRRFWRRGEAC) predicted from the N terminus of the alternatively edited protein-1 from mitochondria of *T. brucei brucei* was purchased from Alpha Diagnostic (San Antonio, TX) (18).

Lipids—All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). These included phosphatidylcholine from egg (PC; 840051), 1,2-distearoyl-*sn*-glycero-3-PC (850365), 1,2-diheptadecanoyl-*sn*-glycero-3-PC (850360), 1,2-dipalmitoyl-*sn*-glycero-3-PC (850355), 1,2-dipentadecanoyl-*sn*-glycero-3-PC (850350), 1,2-dimyristoyl-*sn*-glycero-3-PC (850345), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PC (850457), and 1-stearoyl-2-oleoyl-*sn*-glycero-3-PC (850467).

Trypanosome Killing Assays—BSF *T. brucei brucei* Lister 427 (MiTat 1.2), *T. brucei gambiense* type 1 (Eliane strain), and *T. brucei rhodesiense* KETRI 2482 were used in this study. Trypanosome killing assays were performed as described previously (3, 19, 20). Cultured trypanosomes were incubated in HMI-9 medium (BSF) or SM medium (PCF) at a density of 1×10^7 cells/ml (except for *T. brucei gambiense* cells, which were incubated at 1×10^6 cells/ml) containing either 10% heat-inactivated fetal bovine serum or bovine serum albumin and peptide or control reagents for 2 h at 37 °C. Killing of BSF trypanosomes was evaluated by phase-contrast microscopy. PCF trypanosomes were stained with 0.1% trypan blue. Trypan blue staining was also used for some BSF assays to eliminate any possible discrepancy between the PCF and BSF assays. Trypan blue was added at the end point of each assay to avoid any toxic effects. No difference was observed with the presence of trypan blue in BSF killing assays.

Mammalian Cell Viability and Hemolysis Assays—HEK cells (American Type Culture Collection CRL-1573) and LNCaP prostate cancer cells (CRL-1740) were utilized for cell viability assays. HEK cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Thermo Scientific catalog no. SH30243.01) with 10% fetal bovine serum at 37 °C and 5% CO_2. Prostate cancer cells were cultured in RPMI 1640 medium (Invitrogen catalog no. A10491-01) with 10% fetal bovine serum at 37 °C and 5% CO_2. In both cases, assays were performed by aliquoting cells into 96-well plates at ~60% confluency and allowing the cells to adhere for 2 h. Cells were then incubated with serial dilutions of SHP-1 or the relevant control in the corresponding medium for 2 h at 37 °C. Cell viability was determined by the ability of live cells to exclude trypan blue. The cells were incubated with 0.1% trypan blue for 10 min and examined microscopically for cytoplasmic staining. The potential for SHP-1 to induce hemolysis was assayed by monitoring Hb release from freshly collected human erythrocytes that had been washed and incubated with SHP-1 in PBS. The Hb concentration of the supernatant was determined by the absorbance at 412 nm and compared with 100% hemolysis acquired by hypotonic lysis. The membrane-permeabilizing peptide melittin was used a positive control for killing or hemolysis.

Microscopy—All images were acquired with an Axio Observer Z1 equipped with an AxioCam MRm controlled by AxioVision 4.6 software or a Zeiss Imager A1. Fluorescence microscopy with Texas Red-labeled SHP-1 (Bio-Synthesis, Inc.) was performed by incubating 3×10^6 cells/ml with 8 μM Texas Red-labeled SHP-1 in medium for 10 min before fixing with 1% paraformaldehyde for 1 min, air drying on glass slides, and covering with DAPI containing the antifade reagent ProLong Gold (Molecular Probes). Videos were acquired with live cells at a density of 3×10^6 cells/ml incubated with the indicated concentration of SHP-1 at 37 °C. Movies were recorded at magnification ×63 at 50-ms acquisition times for normal and constricted motion trypanosomes and at 25-ms acquisition times for hyperactivated trypanosomes. Visualized trypanosomes presented in supplemental Movies 1–4 were centered in the videos by digital tracking with Final Cut software (Apple), and videos were looped to facilitate comparisons.

Cell Binding and Uptake Assays—Peptide binding to cells and endocytosis in BSF *T. brucei* were monitored by flow cytometry. Binding assays were performed with 3×10^6 cells/ml in PBS at 25 °C. Texas Red-labeled SHP-1 was added to a final concentration of 0.8 μM, and 50,000 cells were immediately counted with the CyAn ADP flow cytometer (Dako). Fluorescent dextran (Sigma catalog no. FD500S) was utilized to monitor endocytosis at permissive and nonpermissive temperatures. BSF *T. brucei brucei* cells (3×10^6 cells/ml) were preincubated at 37 or 3 °C for 30 min before the addition of either 0.8 μM Texas Red-labeled SHP-1 or 2 mg/ml FITC-labeled dextran. Incubations were continued for 30 min, cells were washed with ice-cold PBS, and 50,000 cells were counted via flow cytometry. Data were analyzed with FlowJo software (TreeStar Inc.).

Calcein Release Assays—Permeabilization of unilamellar liposomes was assayed as described previously (21). Briefly, liposomes were diluted 1:1000 into 50 mM Tris (pH 7.0), and calcein fluorescence was monitored at 513 nm when excited at 484 nm. The percent calcein release was calculated relative to

the 100% fluorescence intensity, achieved by the addition of 0.01% Triton X-100.

Anisotropy Assays—The membrane fluidity of live *T. brucei brucei* was probed by measuring the fluorescence depolarization of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH; Invitrogen catalog no. T204). Cells were washed twice with and resuspended in PBS at a density of 3×10^6 cells/ml. The anisotropic probe TMA-DPH was added to a final concentration of 0.5 μM and allowed to intercalate into the cell membrane for 15 min. Anisotropic values were acquired via the software function of a PerkinElmer Life Sciences LS55 spectrofluorometer. Samples were excited at 358 nm, and emission was read at 430 nm, both with 10-nm slit widths. Data were corrected for light scattering with an unlabeled sample of cells, and anisotropy was calculated according to the equation $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where r is the anisotropy value, I_{VV} is the emission intensity acquired with the excitation- and emission-polarizing filters set vertically, G is the instrument correction factor, and I_{VH} is the emission intensity acquired with the excitation-polarizing filter set vertically and the emission-polarizing filter set horizontally. All assays were conducted at ambient temperature (~25 °C).

RESULTS

SHPs Specifically Kill BSF African Trypanosomes—Our laboratory previously reported that delipidated native human haptoglobin-related protein, purified from high-density lipoproteins, kills BSF *T. brucei brucei* (16, 19). The lack of killing activity in a recombinant form of haptoglobin-related protein that lacks the N-terminal signal peptide (22) led us to investigate the potential trypanocidal activity of a synthetic peptide corresponding to the signal sequence (SHP-1). The addition of SHP-1 to BSF *T. brucei brucei* killed cells in a dose-dependent fashion (Fig. 1*A*). Equivolume amounts of Me_2SO did not exhibit toxicity. The human pathogenic subspecies *T. brucei rhodesiense* and *T. brucei gambiense* were also sensitive to killing by SHP-1 (Fig. 1*A*). Cells treated with lower concentrations of SHP-1 (*i.e.* 4 μM) retained their elongated and twisted shape, and cell membranes appeared completely intact; however, cells were motionless and unable to exclude trypan blue (Fig. 1*B* and data not shown). At higher concentrations of SHP-1 (40 – 80 μM), BSF trypanosomes retained their overall shape; however, cell membranes appeared to have been stripped off or collapsed into the cytoplasm of the cells (Fig. 1*B*). Membrane degradation occurred subsequent to cell death, as revealed by video differential interference contrast (DIC) microscopy (supplemental Movie 4).

SHP-1 Exhibits Specificity for BSF African Trypanosomes—The specificity of SHP-1 was initially examined by conducting killing assays with PCF *T. brucei brucei*. No killing of PCF cells was observed (Fig. 1*A*). Utilizing flow cytometry, we investigated whether the lack of PCF killing was due to a failure of SHP-1 to bind the PCF trypanosome (Fig. 1*C*). Texas Red-labeled SHP-1 rapidly bound BSF *T. brucei* as indicated by the labeling of the entire population of cells immediately after the addition of peptide. No labeling of PCF cells was observed immediately after the addition of peptide,

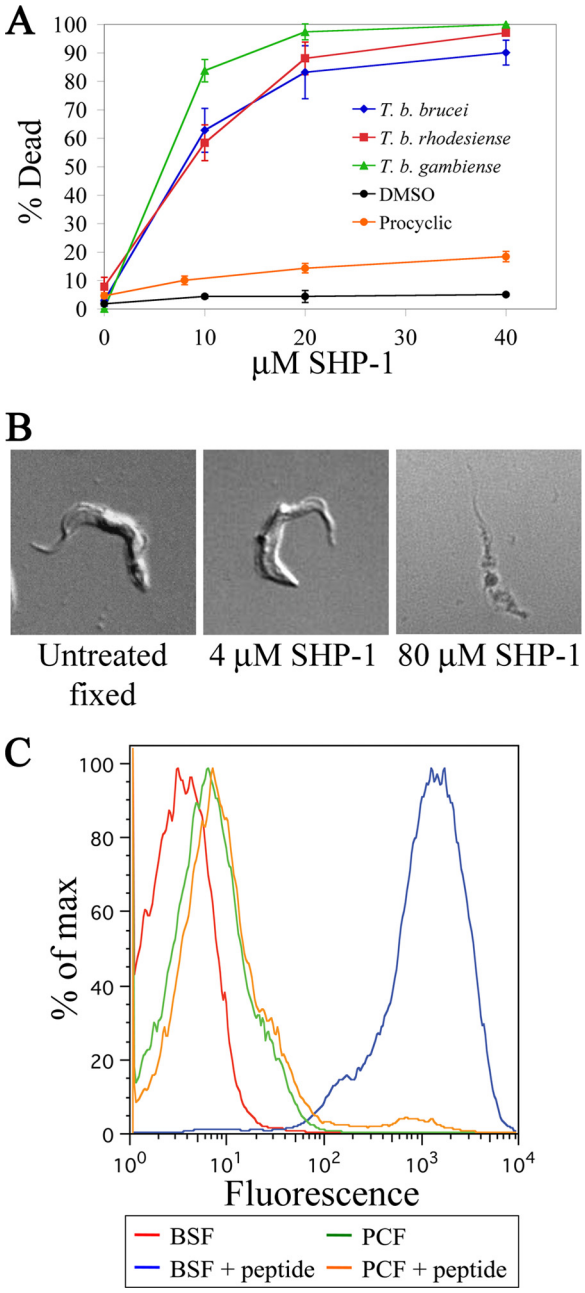

FIGURE 1. **Toxicity and specificity of SHP-1 for BSF African trypanosomes.** *A*, SHP-1 was assayed for killing activity against BSF *T. brucei brucei* (*blue diamonds*), *T. brucei rhodesiense* (*red squares*), and *T. brucei gambiense* (*green triangles*) and PCF *T. brucei brucei* (*orange circles*). Equivolume additions of solvent Me_2SO (*black circles*) showed no toxic effect. *B*, DIC micrographs are shown of untreated *T. brucei brucei* fixed with formaldehyde and unfixed *T. brucei brucei* incubated with 4 and 80 μM SHP-1 for 2 h at 37 °C. *C*, binding of Texas Red-labeled SHP-1 to BSF (without (*red trace*) and with (*blue trace*) peptide) and PCF (without (*green trace*) and with (*orange trace*) peptide) *T. brucei brucei* was monitored by flow cytometry.

indicating a dramatic difference in the affinity of SHP-1 for BSF and PCF trypanosomes.

We broadened our analysis of the spectrum of SHP-1 toxicity by testing the peptide against two human cell lines. Incubating HEK or prostate cancer cells with relatively high concentrations of SHP-1 (80 –160 μM) for 2 h at 37 °C did not result in cell death as evaluated by changes in morphology or the ability to exclude trypan blue (Fig. 2, *A* and *B*). Consistent with a lack of

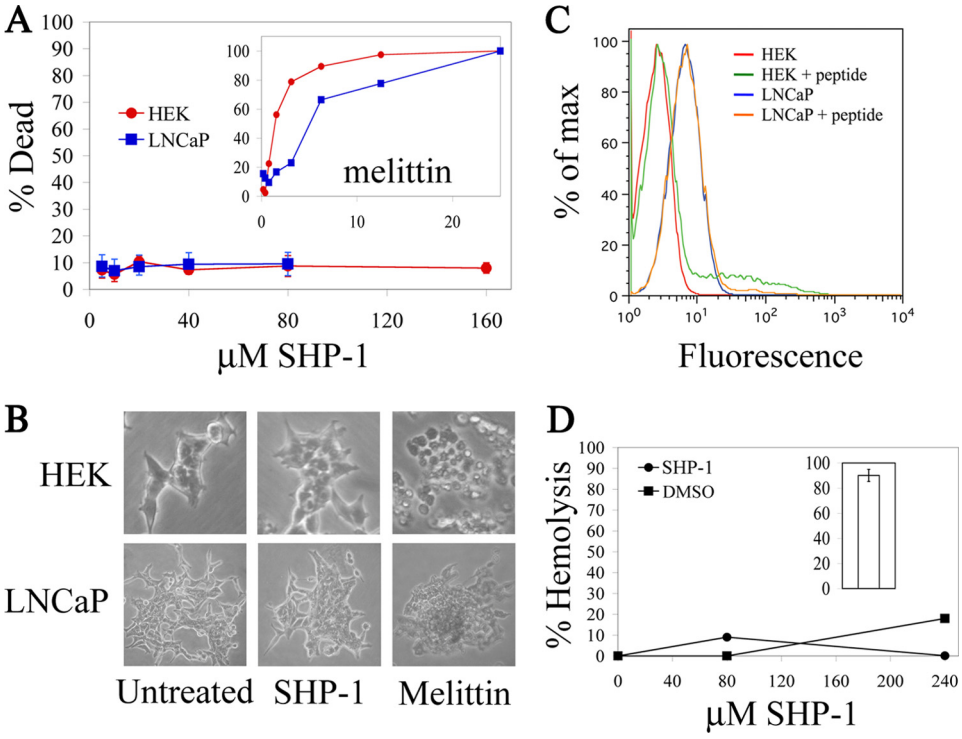

FIGURE 2. **SHP-1 is not toxic to mammalian cells.** *A*, human cell lines HEK (*red circles*) and LNCaP (*blue squares*) were not killed by SHP-1 as assayed by trypan blue exclusion. The cytotoxic peptide melittin was utilized as a positive control (*inset*). *B*, SHP-1 (80 μM) did not induce morphological changes in HEK or LNCaP cells. Melittin (25 μM) was utilized as a positive control. *C*, flow cytometry indicated that Texas Red-labeled SHP-1 did not bind HEK (without (*red trace*) and with (*green trace*) peptide) or LNCaP (without (*blue trace*) and with (*orange trace*) peptide) cells. *D*, the potential hemolytic activity of SHP-1 was assayed against fresh human erythrocytes. *Inset*, 1 μM melittin.

toxicity, flow cytometry indicated that these cells were not bound by SHP-1 (Fig. 2*C*). Additionally, no hemolysis of freshly collected human erythrocytes incubated with 240 μM SHP-1, the highest concentration tested, was detected (Fig. 2*D*).

SHP-1 Killing of BSF Trypanosomes Requires a Hydrophobic Stretch of Amino Acids—The sequence requirements for trypanocidal activity by SHP-1 were analyzed by conducting killing assays with synthetic peptides that mimicked, lessened, or ablated the core hydrophobic region of SHP-1 (Fig. 3*A*). A peptide exhibiting similar hydrophobicity and length was derived from the signal sequence of the human apolipoprotein paraoxonase-1 (SHP-2) (17). We observed trypanocidal activity against BSF *T. brucei* equivalent to SHP-1 (Fig. 3*B*). Conversely, a nonspecific hydrophilic peptide of equal length to SHP-1 exhibited no toxicity (Fig. 3*B*). The size of the hydrophobic core of SHP-1 was decreased by a single leucine deletion from the C-terminal leucine triplicate. Decreasing the length and hydrophobicity by a single amino acid resulted in an ~5-fold decrease in trypanocidal activity (Fig. 3*B*). Further deletions of the C-terminal leucine triplet or the N-terminal Leu-Gly-Ala resulted in a complete lose of trypanosome killing activity (Fig. 3*B*).

The effect of altering peptide hydrophobicity on trypanosome killing suggested that membrane interaction might play a mechanistic role in SHP-1 trypanotoxicity. In an initial attempt to address this possibility, we utilized a model liposome system in which we monitored the release of the internally trapped fluorophore calcein as an indicator of membrane interaction. SHP-1 elicited calcein release from unilamellar liposomes composed of PC from egg at nanomolar concentrations (Fig. 3*C*). Variant SHPs performed in the calcein release assay in a manner consistent with their ability to kill BSF trypanosomes, with a lack of liposome permeabilization by the deletion variants. SHP-2 elicited calcein release, albeit reduced compared with SHP-1, whereas deleting a single leucine, the leucine triplet, or the Leu-Gly-Ala stretch resulted in a loss of permeabilizing activity at a concentration of 500 nM, the highest concentration tested (Fig. 3*C*). These data indicate that (i) a necessary characteristic for trypanosome killing activity is a significantly high degree of hydrophobicity, and (ii) the ability to interact with lipid bilayers is consistent with the ability to kill BSF trypanosomes.

SHP Acts at the Surface of BSF Trypanosomes—Multiple trypanocidal molecules have been identified that exert their toxic effect after localization within an intracellular vesicle rather than at the cell surface (23, 24). Therefore, we asked if SHP-1 requires internalization by the target trypanosome. Killing assays were performed with SHP-1 at 3 °C, a temperature that halts endocytosis (23). We observed robust trypanocidal activity (Fig. 4*A*), equivalent to killing assays performed at 37 °C, suggesting that SHP-1 does not require cellular uptake to exert its toxic effects. Consistent with these data, Texas Red-labeled SHP-1 bound BSF trypanosomes at both 37 and 3 °C (Fig. 4*B*). In contrast, the fluid-phase endocytic cargo FITC-dextran accumulated in BSF trypanosomes at 37 °C but not at 3 °C (Fig. 4*C*). Additionally, Texas Red-labeled SHP-1 exhibited diffuse staining of the entire BSF *T. brucei* cell rather than punctate labeling of endosomes and lysosome exemplified by Alexa 594-labeled transferrin (Fig. 4*D*).

SHP-1/Membrane Interaction Is Dependent on Lipid Bilayer Fluidity—On the basis of the physiological necessity of membrane surface flow in BSF *T. brucei*, we asked what role lipid bilayer fluidity plays in dictating sensitivity to SHP-1. Liposomes composed of highly fluid compositions, such as egg PC (transition temperature (T_m) < 0 °C) (25), were readily permeabilized by SHP-1 (Fig. 5*A*). However, homogeneous liposomes composed entirely of symmetric PC with saturated chains of 15 or 16 carbon atoms (T_m = 34 and 41 °C respectively) (25) were not permeabilized by SHP-1 (Fig. 5*A*). Fluidizing refractory lipid compositions by incorporating increasing mole percent 1,2-dimyristoyl-*sn*-glycero-3-PC (symmetric 14:0, T_m = 23.6 °C) (25) led to a 1,2-dimyristoyl-*sn*-glycero-3-PC concentration-dependent increase in susceptibility to SHP-1 (Fig. 5*B*).

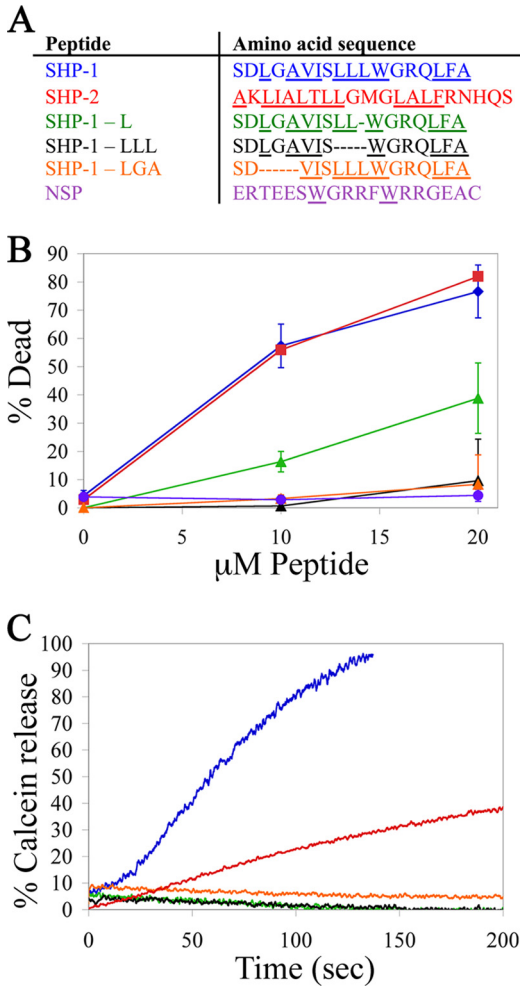

FIGURE 3. **Hydrophobicity requirements for trypanosome killing by small peptides.** *A*, peptide amino acid sequences (from the N to C terminus). Hydrophobic residues are in *boldface* and *underlined*. Colors correspond to descriptions for *B* and *C*. *B*, killing of BSF *T. brucei brucei* by SHP-1 (*blue diamonds*); sequentially distinct SHP-2 (*red squares*); SHP-1 variants SHP-1−L (*green triangles*), SHP-1−LLL (*black triangles*), and SHP-1−LGA (*orange triangles*); and a nonspecific hydrophilic peptide (*NSP*; *purple circles*). *C*, representative traces of liposome permeabilization by 200 nM SHP-1 (*blue trace*); 200 nM SHP-2 (*red trace*); and the deletion variants 500 nM SHP-1−L (*green trace*), 500 nM SHP-1−LLL (*black trace*), and 500 nM SHP-1−LGA (*orange trace*).

To determine whether SHP-1 has a specific affinity for membrane myristate or susceptibility is due to a general physical property imparted by the short acyl chain, we assayed liposomes composed of PC with different acyl chain moieties and transition temperatures. The presence of an unsaturation in 16- or 18-carbon acyl chains results in a large decrease in bilayer transition temperature relative to their unsaturated counterparts. Liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PC (T_m = −2 °C) (25) or 1-stearoyl-2-oleoyl-*sn*-glycero-3-PC (T_m = 6 °C) (25) were susceptible to permeabilization by SHP-1, indicating that myristate is not a requirement for membrane interaction (Fig. 5*C*). We further investigated the role of membrane fluidity in mediating membrane sensitivity to SHP-1 by conducting permeabilization assays with the refractory lipid compositions at temperatures above their T_m values. Fluidizing lipid bilayers composed of 1,2-dipentadecanoyl-*sn*-glycero-3-PC (symmetric 15:0, T_m = 34 °C) or 1,2-dipalmitoyl-*sn*-glycero-3-PC (symmetric 16:0, T_m = 41.3 °C) (25) by bringing liposomal suspensions above their respective T_m values resulted in sensitivity to permeabilization by SHP-1 (Fig. 5*D*). These data indicate that it is not affinity for a specific bilayer moiety but the general physical property of membrane fluidity that confers susceptibility to SHP-1.

SHP-1 Induces Rigidification of BSF Trypanosome Cell Membranes—On the basis of the role of lipid bilayer fluidity in SHP-1/membrane interaction, we asked what effect SHP-1 has upon the fluidity of BSF *T. brucei* membranes. We utilized the surface membrane probe TMA-DPH, a cationic lipophilic molecule that rapidly partitions into the outer leaflet of the cellular lipid bilayer. The addition of SHP-1 increased the rigidity of BSF cell membranes as indicated by an increase in the anisotropy of the surface membrane probe TMA-DPH (Fig. 6*A*). The difference in membrane composition of PCF trypanosomes was apparent in the higher anisotropic values, *i.e.* greater rigidity, acquired for PCF cells. Consistent with the lack of SHP-1 binding to PCF cells and data from model liposomes indicating that a high degree of fluidity is required for SHP-1 intercalation, the anisotropy of PCF trypanosomes was unchanged by the addition of peptide. These data may reflect the physiological differences of BSF and PCF trypanosomes with respect to motility, turnover rates of surface proteins, and endocytic activity.

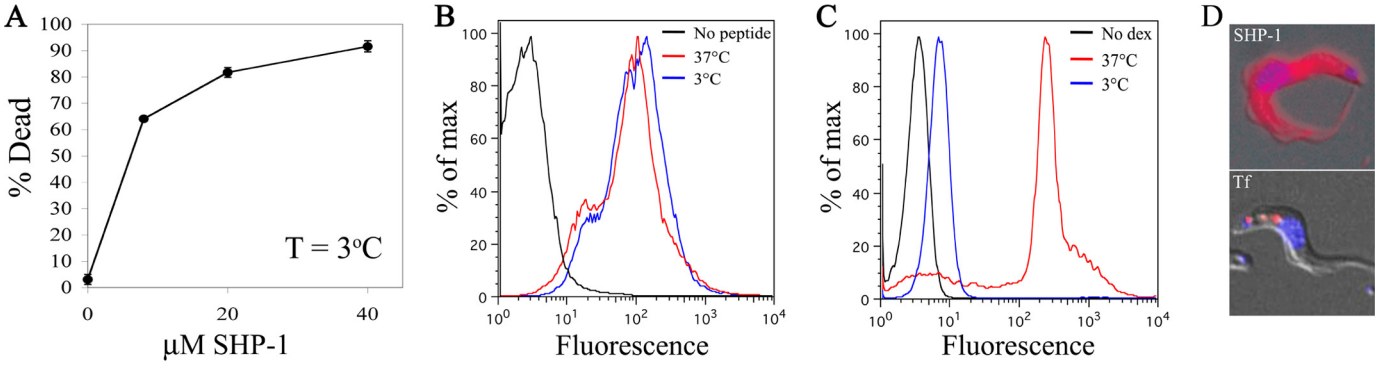

FIGURE 4. **SHP-1 acts at the surface of BSF African trypanosomes.** *A*, killing assays were performed with SHP-1 and BSF *T. brucei brucei* at 3 °C, a temperature nonpermissive for endocytosis. *B*, Texas Red-labeled SHP-1 binding to BSF *T. brucei brucei* at 37 °C (*red trace*) and 3 °C (*blue trace*) was measured by flow cytometry. *Black trace*, no peptide. *C*, the uptake of FITC-dextran at 37 °C (*red trace*) and 3 °C (*blue trace*) was measured by flow cytometry. *Black trace*, no dextran (*dex*). *D*, fluorescence microscopy with Texas Red-labeled SHP-1 revealed diffuse labeling of the cell surface rather than accumulation within an intracellular vesicle as displayed by Alexa 594-labeled transferrin (*Tf*). Cell nuclei and kinetoplast were labeled with DAPI.

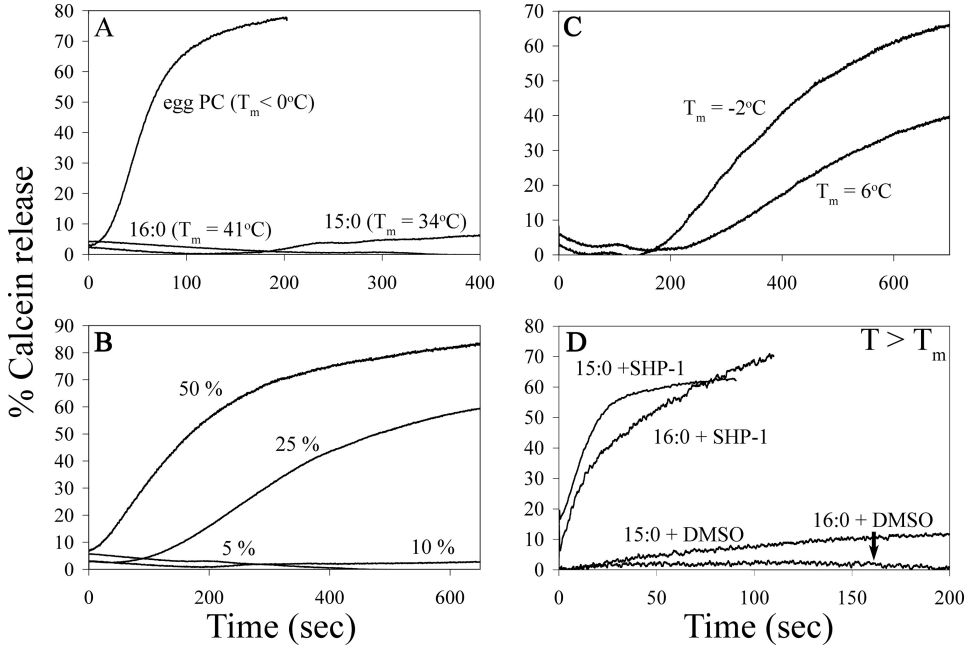

FIGURE 5. **SHP-1 interacts with membranes exhibiting fluid-phase packing.** *A*, lateral van der Waals interactions dictated sensitivity to SHP-1 as indicated by the ability of 200 nM SHP-1 to readily elicit calcein leakage from unilamellar liposomes composed of egg PC (T_m < 0 °C), whereas liposomes composed of homogeneous lipid species with symmetric acyl chains (15:0, T_m = 34 °C; or 16:0, T_m = 41 °C) were resistant. *B*, the addition of 1,2-dimyristoyl-*sn*-glycero-3-PC (indicated by percentage) to refractory compositions (1,2-dipalmitoyl-*sn*-glycero-3-PC) rendered liposomes susceptible to permeabilization by SHP-1. *C*, liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PC or 1-stearoyl-2-oleoyl-*sn*-glycero-3-PC (asymmetrical PC lacking myristate but exhibiting fluid lipid packing due to unsaturations (16:0 and 18:1, T_m = −2 °C; and 18:0 and 18:1, T_m = 6 °C)) were susceptible to permeabilization by SHP-1. *D*, thermal fluidization of symmetrical 15:0 and 16:0 PC lipid bilayers rendered liposomes susceptible to permeabilization by SHP-1. Assays were carried out at ~60 °C.

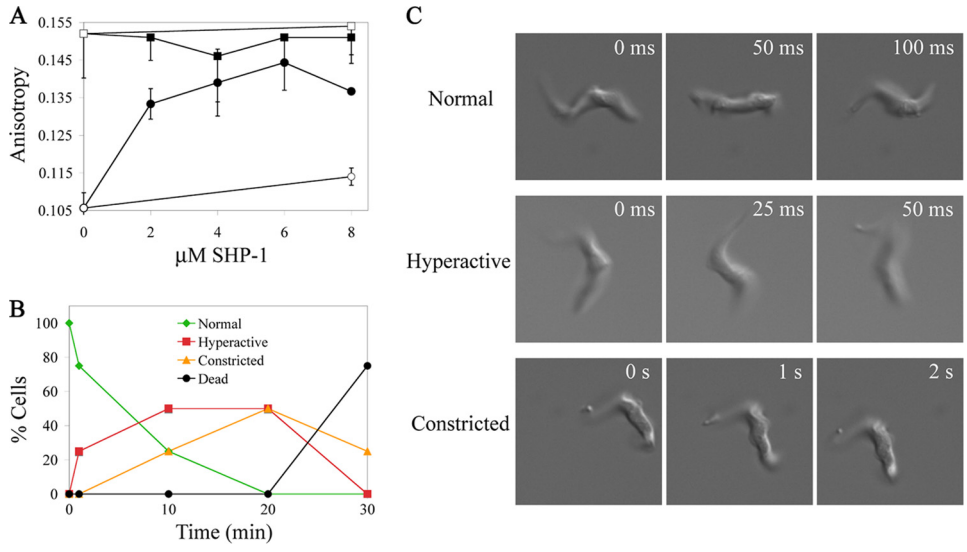

FIGURE 6. **SHP-1 changes the mechanoelastic properties of BSF *T. brucei brucei* membranes and induces dramatic changes in motility.** *A*, the rigidity of BSF *T. brucei* plasma membranes was increased by the addition of SHP-1 as revealed by the anisotropic changes in the membrane probe TMA-DPH. *Closed circles*, SHP-1; *open circles*, Me_2SO only. SHP-1 had no effect on the anisotropy of PCF *T. brucei* plasma membranes. *Closed squares*, SHP-1; *open squares*, Me_2SO only. *B*, the graph depicts the changes in motility of BSF *T. brucei* treated with 8 μM SHP-1. *C*, images captured via video DIC microscopy illustrate the normal, hyperactive, and constricted phenotypes of cells treated with SHP-1 (supplemental Movies 1–3, respectively).

SHP-1 Induces Dramatic Changes in Cell Motility—During the course of our investigations on BSF *T. brucei* killing, it became readily apparent that treatment of cells with SHP-1 resulted in motility changes. To assess the effect of SHP-1 on the motility of trypanosomes, we analyzed BSF cells by video DIC microscopy. The addition of 8 μM SHP-1 resulted in a progression of motility changes over 30 min (Fig. 6*B*). Untreated BSF trypanosomes exhibited a characteristic corkscrew motion and appeared to accomplish a full rotation within 100 ms (Fig. 6*C* and supplemental Movie 1). Within 1 min of SHP-1 addition, a significant fraction of cells exhibited hyperactive motility, displaying an apparent rotation frequency of 1.5–2/50 ms (Fig. 6*C* and supplemental Movie 2). Hyperactivated cells were still present at 10–20 min; however, a portion of the cells displayed a highly constricted motion, commonly seen as a bent or boomerang-shaped cell that failed to complete a rotation (Fig. 6*C* and supplemental Movie 3). The constricted motility phenotype was increasingly apparent at 20 min, with ~50% of the cells displaying constriction and the other 50% remaining hyperactivated in their motion. During the time course, dead cells were increasingly apparent. The changes in motility and onset of death were more rapid at 80 μM SHP-1, with all of the cells exhibiting hyperactivity until slowing down and dying within 10 min.

DISCUSSION

We have derived a small peptide that rapidly intercalates into the plasma membrane of BSF *T. brucei* and induces cell death. The peptide is highly specific for the developmental form found within a mammalian host. Specificity is mediated at the level of binding or, more likely, intercalation into the acyl chain region of the target membrane. Indeed, studies with model membrane systems indicate that the peptide is sensitive to the acyl chain composition of lipid bilayers. In particular, the membrane fluidity that is imparted by a given acyl chain composition dictates the ability of this peptide to interact with membranes.

Killing of BSF *T. brucei* has been demonstrated for a variety of *bona fide* antimicrobial peptides (15, 27), such as the cathelicidins and their derivatives novispirin and ovispirin, as well as unusual candidates such as neuropeptides (24). In the case of

cathelicidins, it has been shown that, as is the case for their prokaryotic targets, these peptides permeabilize the plasma membrane and that cells assume a rounded and crumpled morphology upon death (15). At relatively high concentrations of SHP-1, we see massive disruption of the cell membrane; however, as revealed by video microscopy (supplemental Movie 4), this occurs subsequent to cell death. Treatment with lower concentrations of SHP-1 (*i.e.* 4 μM) results in dead *T. brucei* cells that exhibit a normal morphology, and DIC microscopy indicates that the cell membrane remains intact. The lack of osmotic swelling suggests that permeabilization of the plasma membrane is not the mechanism of killing. Furthermore, the observed changes in BSF *T. brucei* cell membrane rigidity by SHP-1 suggest a novel mechanism of toxicity.

BSF African trypanosomes are highly dynamic cells with respect to motility (4, 5), endocytosis and vesicle trafficking (6), and lateral flow of surface molecules (7, 9). All of these activities require movement of membrane components such as phospholipids and, in the case of endocytosis and vesicle trafficking, remodeling of the lipid bilayer. Decreasing the physical property of fluidity in the bulk membrane such that these activities are burdened may contribute to a general poisoning of the cell by SHP-1. Alternatively, or in synergy with membrane rigidification, SHP-1 may act through nonspecific alterations of integral membrane protein stability/activity (28). For example, distortion of the lateral pressure profile, a mechanism that has been attributed to general anesthetics (29), may lead to increased lateral pressure, which in turn may physically inhibit transmembrane channels (30). Additionally, peptides corresponding to the *Escherichia coli* LamB signal peptide have been shown to affect the oligomerization of integral transmembrane proteins (31). Such surface activity is consistent with the SHP-1 site of action being the plasma membrane, as suggested by killing assays performed at 3 °C (Fig. 4*A*) and labeling of the surface of BSF *T. brucei* by Texas Red-SHP-1 (Fig. 4*B*). In either case, the fluidity of the BSF trypanosome cell membrane appears not only to be modified by SHP-1 but also to be specifically targeted.

Our studies with model liposomes indicate that the fluidity of the target lipid bilayer determines the ability of SHP-1 to bind and permeabilize membranes. These data are consistent with previous studies showing that modulation of the surface pressure of lipid monolayers dictates the ability of signal peptides to intercalate into the acyl chain region (32). Membranes exhibiting rigid gel-phase lipid order are refractory to permeabilization by SHP-1. When these refractory liposomes are brought above their transition temperatures, thus exhibiting fluid liquid crystalline order, they are rendered susceptible to permeabilization by SHP-1. These data indicate that the lateral van der Waals forces dictate the ability of SHP-1 to intercalate into target lipid environments. The immediate labeling of the entire population of BSF *T. brucei* by Texas Red-labeled SHP-1 indicates a very rapid rate of bilayer intercalation (Fig. 1*C*).

The consequences of SHP-1 intercalation into BSF trypanosome membranes are immediately apparent on the cellular level. Cells exhibit hypermotility immediately after introduction of SHP-1, subsequently becoming constricted in their motion before dying. The cause for these changes in motility can only be speculated upon. The change in overall mechanoelastic properties of the cell may be directly responsible. Spermatozoa are examples of flagellated cells that undergo changes in membrane fluidity concurrent with the onset of hypermotility (33). In these cells, hyperactivation is associated with an efflux of cholesterol and an increase in membrane fluidity. Another possibility is that the peptide induces changes in the distribution of membrane components. In this regard, Tyler *et al.* (34) have demonstrated recently that *T. brucei* flagellar membranes are enriched in cholesterol and exhibit a higher degree of order than the pellicle of the cell and that flagellar lipid components can redistribute throughout the cell membrane in response to lipid bilayer-modifying agents. It is intriguing to imagine that SHP-1 induces a redistribution of cholesterol, sphingomyelin, or other components from the flagellum to the pellicular fraction of the cell membrane, in turn resulting in an overall increase in cell membrane rigidity coupled with a localized decrease in the flagellar rigidity and thus alleviation of physical strain against flagellar motors and acceleration of flagellar beating. The observed constricted motility at later time points following treatment with SHP-1 might be the result of metabolic depletion.

Currently, there are ~70,000 people infected with African trypanosomes mainly in four sub-Saharan nations (World Health Organization). Treatment of infected individuals is limited, and there is an increase in the number of relapses after established drug treatment (35). Few new drugs are currently under clinical trials, and one of the more promising compounds, DB75, has already shown a marked tendency to induce drug resistance (36). SHPs target a fundamental physiological characteristic of *T. brucei*, namely the fluid physical property of the cell membrane. It is not likely that BSF trypanosomes will quickly develop a strategy to circumvent the cytotoxicity of molecules that alter the mechanoelastic properties of the cell membrane. Therefore, we suggest that the BSF trypanosome cell membrane is an attractive target for the development of novel therapeutics.

Acknowledgments—We thank all members of the Hajduk laboratory as well as Robert Sabatini and Laura Cliffe for valuable discussions and Tim Dowse for video editing.

REFERENCES

1. Barrett, M. P., Burchmore, R. J., Stich, A., Lazzari, J. O., Frasch, A. C., Cazzulo, J. J., and Krishna, S. (2003) *Lancet* **362,** 1469–1480
2. Rifkin, M. R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3450–3454
3. Hajduk, S. L., Moore, D. R., Vasudevacharya, J., Siqueira, H., Torri, A. F., Tytler, E. M., and Esko, J. D. (1989) *J. Biol. Chem.* **264,** 5210–5217
4. Rodríguez, J. A., Lopez, M. A., Thayer, M. C., Zhao, Y., Oberholzer, M., Chang, D. D., Kisalu, N. K., Penichet, M. L., Helguera, G., Bruinsma, R., Hill, K. L., and Miao, J. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 19322–19327
5. Oberholzer, M., Lopez, M. A., McLelland, B. T., and Hill, K. L. (2010) *PLoS Pathog.* **6,** e1000739
6. Engstler, M., Thilo, L., Weise, F., Grünfelder, C. G., Schwarz, H., Boshart, M., and Overath, P. (2004) *J. Cell Sci.* **117,** 1105–1115
7. Bülow, R., Overath, P., and Davoust, J. (1988) *Biochemistry* **27,** 2384–2388
8. Field, M. C., and Carrington, M. (2009) *Nat. Rev.* **7,** 775–786
9. Engstler, M., Pfohl, T., Herminghaus, S., Boshart, M., Wiegertjes, G., Heddergott, N., and Overath, P. (2007) *Cell* **131,** 505–515

10. Ferguson, M. A., Low, M. G., and Cross, G. A. (1985) *J. Biol. Chem.* **260,** 14547–14555
11. Ferguson, M. A., Haldar, K., and Cross, G. A. (1985) *J. Biol. Chem.* **260,** 4963–4968
12. Langreth, S. G., and Balber, A. E. (1975) *J. Protozool.* **22,** 40–53
13. Morgan, G. W., Allen, C. L., Jeffries, T. R., Hollinshead, M., and Field, M. C. (2001) *J. Cell Sci.* **114,** 2605–2615
14. Field, M. C., Menon, A. K., and Cross, G. A. (1991) *EMBO J.* **10,** 2731–2739
15. McGwire, B. S., Olson, C. L., Tack, B. F., and Engman, D. M. (2003) *J. Infect. Dis.* **188,** 146–152
16. Smith, A. B., Esko, J. D., and Hajduk, S. L. (1995) *Science* **268,** 284–286
17. Sorenson, R. C., Bisgaier, C. L., Aviram, M., Hsu, C., Billecke, S., and La Du, B. N. (1999) *Arterioscler. Throm. Vasc. Biol.* **19,** 2214–2225
18. Ochsenreiter, T., and Hajduk, S. L. (2006) *EMBO Rep.* **7,** 1128–1133
19. Shiflett, A. M., Bishop, J. R., Pahwa, A., and Hajduk, S. L. (2005) *J. Biol. Chem.* **280,** 32578–32585
20. Widener, J., Nielsen, M. J., Shiflett, A., Moestrup, S. K., and Hajduk, S. (2007) *PLoS Pathog.* **3,** 1250–1261
21. Harrington, J. M., Howell, S., and Hajduk, S. L. (2009) *J. Biol. Chem.* **284,** 13505–13512
22. Vanhollebeke, B., Nielsen, M. J., Watanabe, Y., Truc, P., Vanhamme, L., Nakajima, K., Moestrup, S. K., and Pays, E. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 4118–4123
23. Hager, K. M., Pierce, M. A., Moore, D. R., Tytler, E. M., Esko, J. D., and Hajduk, S. L. (1994) *J. Cell Biol.* **126,** 155–167
24. Delgado, M., Anderson, P., Garcia-Salcedo, J. A., Caro, M., and Gonzalez-Rey, E. (2009) *Cell Death Differ.* **16,** 406–416
25. Koynova, R., and Caffrey, M. (1998) *Biochim. Biophys. Acta* **1376,** 91–145
26. Deleted in proof
27. Haines, L. R., Thomas, J. M., Jackson, A. M., Eyford, B. A., Razavi, M., Watson, C. N., Gowen, B., Hancock, R. E., and Pearson, T. W. (2009) *PLoS Negl. Trop. Dis.* **3,** e373
28. Lee, A. G. (2004) *Biochim. Biophys. Acta* **1666,** 62–87
29. Cantor, R. S. (1998) *Toxicol. Lett.* **100–101,** 451–458
30. Kamaraju, K., and Sukharev, S. (2008) *Biochemistry* **47,** 10540–10550
31. Benach, J., Chou, Y. T., Fak, J. J., Itkin, A., Nicolae, D. D., Smith, P. C., Wittrock, G., Floyd, D. L., Golsaz, C. M., Gierasch, L. M., and Hunt, J. F. (2003) *J. Biol. Chem.* **278,** 3628–3638
32. Briggs, M. S., Cornell, D. G., Dluhy, R. A., and Gierasch, L. M. (1986) *Science* **233,** 206–208
33. Visconti, P. E., Westbrook, V. A., Chertihin, O., Demarco, I., Sleight, S., and Diekman, A. B. (2002) *J. Reprod. Immunol.* **53,** 133–150
34. Tyler, K. M., Fridberg, A., Toriello, K. M., Olson, C. L., Cieslak, J. A., Hazlett, T. L., and Engman, D. M. (2009) *J. Cell Sci.* **122,** 859–866
35. Brun, R., Schumacher, R., Schmid, C., Kunz, C., and Burri, C. (2001) *Trop. Med. Int. Health* **6,** 906–914
36. Lanteri, C. A., Stewart, M. L., Brock, J. M., Alibu, V. P., Meshnick, S. R., Tidwell, R. R., and Barrett, M. P. (2006) *Mol. Pharmacol.* **70,** 1585–1592