

DEVELOPMENTALLY REGULATED,
PHOSPHOLIPASE C-MEDIATED RELEASE OF
THE MAJOR SURFACE GLYCOPROTEIN OF
AMASTIGOTES OF *TRYPANOSOMA CRUZI*

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Many eukaryotic proteins are anchored to the membrane by glycosyl-phosphatidylinositol (GPI)¹ (1, 2). Included among them are the major surface glycoproteins of two members of the *Trypanosomatidae* family: the variant surface glycoprotein (VSG) of African trypanosomes (3, 4) and the *Leishmania* surface protease (5).

cDNA sequencing of GPI-anchored proteins has revealed predicted COOH-terminal hydrophobic peptide sequences (6–8) that are absent from the mature protein (6, 7). These residues are replaced by the glycolipid tail in an early posttranslational event. The rapidity of the process of tail addition (9) suggests that a preformed unit is added by a transpeptidase reaction; a glycolipid precursor has indeed been identified in *T. brucei* (10). The functional significance of the substitution of a conventional anchor sequence by this unusual glycolipid remains obscure. The presence in *T. brucei* of a phospholipase C which specifically hydrolyzes the anchor suggests that this structural feature is designed to permit the rapid and selective release of these surface proteins (4). However, direct evidence for a physiological role of the GPI-specific phospholipase C in *T. brucei* is lacking.

We have previously shown that the transformation of trypomastigotes of *T. cruzi* into amastigotes is associated with the expression of Ssp-4, a stage-specific surface antigen (11). Here we present evidence that Ssp-4 is the major surface glycoprotein of amastigotes and that it is anchored to the membrane by GPI. We also show that in vitro Ssp-4 is progressively shed from amastigotes during their development into epimastigotes. Because large numbers of amastigotes can be easily cultivated in medium for several days in the absence of mammalian cells

This work was supported by grants from the MacArthur Foundation and the United Nations Development Program/World Bank/World Health Organization. N. Andrews was supported by a Research Training Grant from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases. V. Ley is a fellow from Fundacion Juan March, Spain. K. S. Hong's current address is the Department of Bacteriology, Osaka University School of Medicine, Institute of Microbial Diseases, Osaka 565, Japan.

¹ *Abbreviations used in this paper:* CRD, cross-reactive determinant; GPI, glycosyl-phosphatidylinositol; LIT, liver infusion tryptose; VSG, variant surface glycoprotein.

without appreciable loss in viability, we reasoned that this experimental model might be used to study mechanisms of release of a GPI-anchored protein.

Materials and Methods

Parasites. Epimastigotes from the Y strain were grown at 28°C in liver infusion tryptose (LIT) medium (12). Cell culture trypomastigote forms from the Y strain were maintained in monolayers of LLC-MK₂ cells. These epithelial cells were plated on glass coverslips at a density of 2×10^4 cells/cm², in DME containing 10% FCS (Gibco, Grand Island, NY). After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, the cells were exposed to a suspension of 5×10^6 trypomastigotes/ml in DME containing 2% FCS. 3 h later the coverslips were washed three times in HBSS (Gibco) and reincubated for varying periods of time in DME with 2% FCS. Trypomastigotes released from the infected cells were incubated in LIT medium at 37°C for 24–48 h for extracellular transformation into amastigotes (11).

Monoclonal Antibodies. These were prepared as described in reference 11. The mAbs used in this study, 2C2 (IgG 2a) and 3A10 (IgG 1), were purified by ion exchange chromatography. 2C2 was labeled with biotin (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Preparation of an Antiserum to Ssp-4. In vitro–developed amastigote forms (10⁹) were lysed in 100 µl of 10% Tween 20 (Sigma Chemical Co., St. Louis, MO), 10 mM Tris, pH 9.0, and the insoluble material was removed by centrifugation. The supernatant was diluted to contain 0.5% Tween 20 (final volume 2 ml) in the same buffer and applied to a TSK-DEAE-5 PW column equilibrated in 10 mM Tris, pH 9.0, containing 0.05% Tween 20. Ssp-4 was eluted with an FPLC system (LKB Instruments, Inc., Bromma, Sweden) by application of a linear concentration gradient of sodium chloride (0–0.75 M) at a flow rate of 1 ml/min. Ssp-4 was assayed by Western blotting using the 2C2 mAb and anti-cross-reactive determinant (CRD) antibodies. Two fractions, which after SDS-PAGE showed a single silver-stained (Bio-Rad Laboratories, Richmond, CA) band of 80,000 M_r, were pooled, emulsified with CFA (Difco Laboratories, Inc., Detroit, MI), and injected into the footpad of a rabbit. The rabbit was bled 15 d later and the IgG fraction was purified from the serum by DEAE–Sephrose chromatography.

Indirect Immunofluorescence. Infected LLC-MK₂ cell monolayers were fixed in absolute methanol for 15 min at 4°C. Free parasites were fixed in PBS containing 2% formaldehyde and were air dried on glass slides. These preparations were washed with PBS and incubated for 30 min at 25°C with the 2C2 mAb at 50 µg/ml in PBS containing 1% BSA. After three washes in PBS, a FITC-labeled affinity-purified goat antibody to mouse IgG and IgM (Kierkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added. After 30 min of incubation at 25°C, the slides were rinsed in PBS and mounted in 10% glycerol, 0.1 M sodium bicarbonate, pH 9.0, and observed with a fluorescence microscope.

Surface Radiolabeling. In vitro–developed amastigote forms were washed three times in 199 medium (Gibco) and labeled with ¹²⁵I using Iodogen, according to the manufacturer's instructions (Pierce Chemical Co.).

Immunoprecipitation. 10⁶/µl ¹²⁵I-labeled amastigotes were lysed in either 1% NP-40 (Sigma Chemical Co.) in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA at room temperature, or in 1% SDS in 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, at 100°C. Both lysis buffers contained 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF (Sigma Chemical Co.). SDS lysates were sonicated for 10 min and diluted to 0.02% in NP-40 lysis buffer, and supernatant culture medium was centrifuged at 100,000 g for 5 min before overnight incubation at 4°C with antibodies: the specific or unrelated mAbs, mouse anti-*T. cruzi* serum (11), normal rabbit serum, or rabbit antibodies against the CRD of *T. brucei* (a gift from Dr. David Russell, New York University). When lysates and supernatants were being analyzed in parallel, LIT medium or detergent was added in order to obtain identical conditions for immunoprecipitation. After 1 h of incubation with 20 µl of a 25% suspension (wt/vol) of protein A–Sephrose (Zymed Laboratories, San Francisco, CA) the precipitates were washed as described previously (Andrews et al., 1984) and analyzed by SDS-PAGE

after elution in 1% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromophenol blue, and 10% 2-ME.

Two-dimensional PAGE. The protein A-Sepharose-bound immunoprecipitates were eluted in 9.5 M urea, 2% NP-40, 2% ampholines (1.8% 5/7 and 0.2% 3/10), and 5% 2-ME, and submitted to isoelectric focusing under equilibrium conditions (13). The second dimension was run in 10% polyacrylamide slab gels. All reagents were purchased from Bio-Rad Laboratories. Molecular mass markers (Sigma Chemical Co.) were myosin (205 kD), β -galactosidase (116 kD), phosphorylase B (97 kD), BSA (66 kD), OVA (45 kD), and carbonic anhydrase (29 kD).

Con A Binding Assay. NP-40 1% lysates of ^{125}I surface-labeled amastigotes were loaded into Sepharose-Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) columns preequilibrated in PBS containing 2% NP-40. The bound material was eluted with a mixture of 0.1 M α -methyl-D-mannoside and 0.1 M α -methyl-D-glucoside (Sigma Chemical Co.) in PBS 2% NP-40. Bound and unbound fractions were then analyzed by SDS-PAGE.

Metabolic Labeling with [^3H]Myristic Acid. 6×10^7 ml trypomastigotes were incubated overnight at 37°C in LIT medium containing 150 $\mu\text{Ci/ml}$ 9,10 [^3H]myristic acid (22.4 Ci/mmol; New England Nuclear, Boston, MA) complexed with defatted BSA. During this period the trypomastigotes changed into amastigotes, which were then washed three times with 199 medium, resuspended in PBS, and added to an equal volume of boiling 1% SDS, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA. A sample of this lysate was diluted 20 times with PBS and extracted with an equal volume of H_2O -saturated butanol for the determination of the total incorporated radioactivity. A volume equivalent to the lysate of 1×10^8 parasites was diluted to 0.02% SDS, 1% NP-40, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, and immunoprecipitated after overnight incubation with 40 μg of the 2C2 or IA10 mAbs. In a dual labeling experiment, 1×10^8 trypomastigotes were resuspended in 1 ml of LIT medium containing 250 μCi 9,10 [^3H]myristic acid or [^{35}S]methionine-cysteine (70% methionine, 20% cysteine mixture, Tran [^{35}S] label, ICN Radiochemicals, Costa Mesa, CA; 1,122 Ci/mmol) and incubated at 37°C for 48 h. The labeled parasites (now amastigotes) were washed and lysed in boiling SDS, and the supernatants were subjected to 5 min of centrifugation at 100,000 g. Parasite extracts and supernatants were then immunoprecipitated under the same conditions as described above. The precipitates were solubilized by boiling in SDS-PAGE sample buffer, diluted 10 times in 10 mM Tris-HCl, pH 7.5, extracted three times with an equal volume of H_2O -saturated butanol, and the radioactivity associated with the aqueous phase was quantitated in a scintillation counter.

Triton X-114 Partition. 25 μl of 1% SDS, or 1% Triton X-100 lysates of ^{125}I -labeled amastigotes, or 68 μl of LIT culture medium from the radiolabeled amastigotes were mixed with recondensed Triton X-114 (14) (Sigma Chemical Co.) in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, at a final concentration of 2%, at 0°C. LIT medium and detergent were added to the samples to obtain identical conditions, and a final volume of 525 μl . Three successive partitions were performed by incubating the lysates at 37°C for 5 min and adding more Triton X-114 or buffer to the upper and lower phases, respectively (14). Samples of the aqueous and detergent-enriched phases were diluted to 0.1% Triton X-114 with 1% Triton X-100, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, incubated with mAbs, anti-CRD antibodies, or normal rabbit serum, immunoprecipitated, and analyzed by SDS-PAGE. The gels were dried, and after autoradiography the sections corresponding to the migrating position of Ssp-4 were excised and counted in a gamma counter.

Viability Assays. These were performed by microscopically counting fluorescent cells after incubation with fluorescein diacetate and ethidium bromide (15).

FACS Analysis. After different periods of extracellular incubation at 37°C, the parasites were resuspended in PBS with 1% BSA and fixed by addition of an equal volume of 2% paraformaldehyde in PBS, at 0°C. For each time point 12×10^6 cells were centrifuged, resuspended in 100 μl PBS containing 1% BSA, and incubated with 2 $\mu\text{g/ml}$ biotinylated 2C2 for 30 min at room temperature. The parasites were then washed with PBS containing 1% BSA and incubated for 30 min at room temperature in 200 μl Streptavidin-Phycoerythrin conjugate (Becton Dickinson & Co., Oxnard, CA). After washing and resuspension

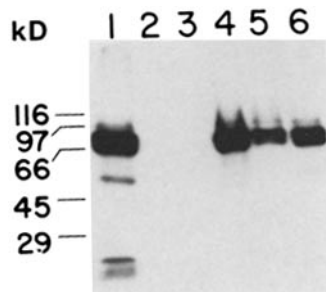


FIGURE 1. SDS-PAGE analysis of Ssp-4 under reducing conditions. Lysates of ^{125}I surface-labeled amastigotes obtained after 36 h of extracellular incubation were immunoprecipitated with mAbs or with a mouse anti-*T. cruzi* serum. (Lane 1) total lysate; (lane 2), 2H11 (specific for Ssp-1, a glycoprotein from the membrane of trypomastigotes of *T. cruzi*); (lane 3), 1A10 (unrelated, against human decay-accelerating factor); (lane 4), mouse anti-*T. cruzi*; (lane 5) 2C2 (Ssp-4 specific); (lane 6) 3A10 (Ssp-4 specific).

in 1 ml PBS containing 0.1% BSA, the cells were analyzed in an Ortho 50H cytofluorograph interfaced with an Ortho 2150 computer.

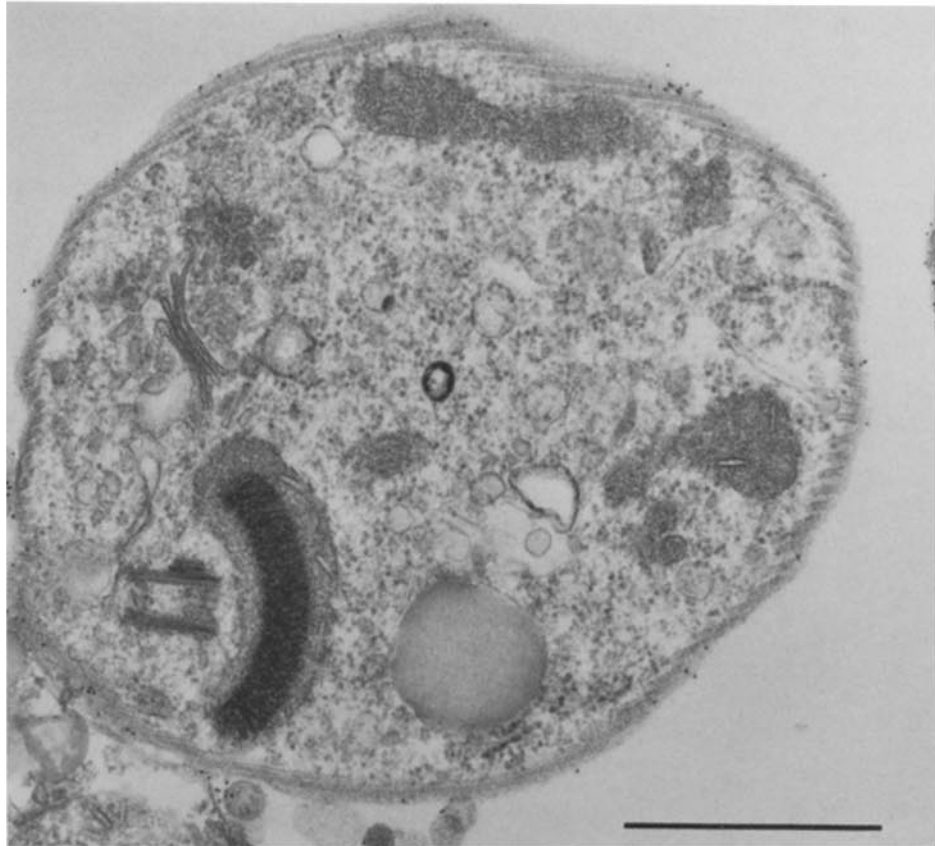
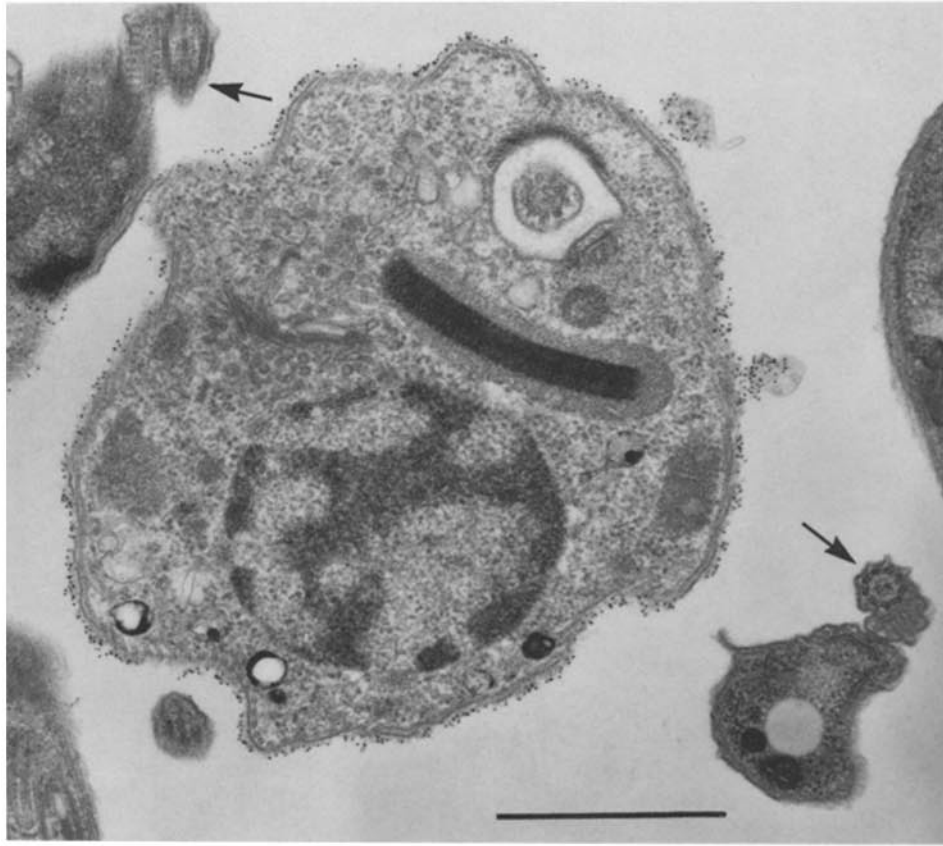
Localization of Ssp-4 by Immunogold Labeling. Trypomastigotes recently emerged from mammalian cells in culture were incubated in LIT medium at 37°C (50×10^6 parasites in 2 ml). After 6 and 48 h the parasites were washed three times in 199 medium and incubated for 1 h in PBS/1% BSA containing 50 $\mu\text{g}/\text{ml}$ of purified IgG from normal rabbit serum or from rabbit antiserum to Ssp-4. After three more washings, protein A complexed to 10 nm colloidal gold (AutoProbe EM protein A A G10; Janssen, Beerse, Belgium) diluted 1:5 in PBS/1% BSA was added for 1 h, and the cells were washed again. The labeling procedure was done at 4°C. The parasites were pelleted and fixed for 2 h at 4°C with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose. After three 0.1-M cacodylate buffer rinses, the pellets were osmicated in 2% OsO_4 in the same buffer, dehydrated in ethyl alcohol, and embedded in Epon 812. Thin sections were picked up on formvar-coated carbon-stabilized grids, stained with 1% aqueous uranyl acetate and 2% lead citrate, and examined in a Philips 300 transmission electron microscope.

Kinetic Studies of Ssp-4 Release. Trypomastigotes were allowed to develop in LIT medium for 24 h. The recently differentiated amastigotes were washed and surface labeled with ^{125}I . 6×10^7 parasites were then resuspended in 1.5 ml of LIT medium and samples of 250 μl were taken after 0, 10, 24, and 48 h of continued incubation in a 5% CO_2 atmosphere at 37°C. At each time point the parasites were collected by centrifugation (1,000 g) and immediately lysed in boiling SDS. The corresponding culture medium supernatants were centrifuged at 100,000 g for 5 min to remove particulate materials. Parasite lysates and culture medium were immunoprecipitated with excess of either 2C2 mAb or anti-CRD antibodies, as described above. Quantitation of the amounts of Ssp-4 precipitated at each time point was done by counting the corresponding bands excised from SDS-PAGE gels.

Lipase Assay. The [^3H]myristate-labeled membrane form of VSG was prepared as described in reference 16. *T. cruzi* extracts were prepared by lysing 5×10^7 parasites in 1 ml of 1% NP-40, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, and removing the insoluble fraction by centrifugation. For the lipase activity assays, the extracts were incubated for 30 min at 37°C with 4 μg of [^3H]myristate-labeled VSG ($\sim 2,500$ cpm), in a total volume of 100 μl of a buffer containing 1% NP-40, 0.04% SDS, 10 mM Tris, pH 8.0, and 5 mM EDTA. The release of [^3H]myristate-labeled dimyristoyl glycerol from VSG was measured by liquid scintillation counting of *n*-butanol extracts of the reaction mixture, and the reaction products were analyzed by silica gel TLC (16).

Results

Ssp-4 is the Major Surface Glycoprotein of Amastigotes. Total lysates of ^{125}I surface-labeled amastigotes contained two major labeled polypeptides with M_r 's of 70,000 and 84,000 under both reducing (Fig. 1, lane 1) or nonreducing conditions. After immunoprecipitation with Ssp-4-specific mAbs 2C2 and 3A10 (lanes 5 and 6) and with a mouse antiserum to *T. cruzi* (lane 4), the same two



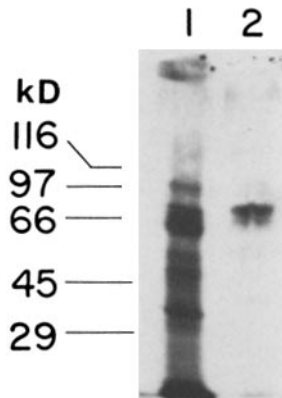


FIGURE 3. [^3H]Myristic acid labeling of Ssp-4. Amastigotes of *T. cruzi* were labeled with [^3H]myristic acid and lysed in boiling SDS. Shown are the SDS-PAGE fluorography patterns. (Lane 1) Total amastigote lysate; (lane 2) immunoprecipitate obtained with the 2C2 mAb.

bands were revealed. Immunoprecipitation with an excess of the 2C2 mAb removed the bands reacting with the polyclonal antibodies (not shown). As controls, the mAbs 2H11 (specific for the Ssp-1 glycoprotein of trypomastigote forms [reference 11]) and IA10 (unrelated) were used for immunoprecipitations under the same conditions, with negative results (lanes 2 and 3).

Ssp-4 is a glycoprotein: it was retained when amastigote lysates were filtered through a column containing immobilized Con A and was eluted with specific sugars (not shown). As shown by colloidal gold immunolabeling and transmission EM, Ssp-4 forms a homogeneous layer around the surface of the amastigotes. Trypomastigote forms were not labeled (Fig. 2, *top*).

Ssp-4 Is Anchored to the Membrane by Glycosyl-phosphatidylinositol. Amastigotes were obtained by incubation of trypomastigotes at 37°C for 24–48 h in medium in the absence of mammalian cells (11). When the parasites were cultivated in the presence of [^3H]myristic acid, the radioactivity was incorporated into several macromolecules (Fig. 3, lane 1). The lipid-labeled Ssp-4 was immunoprecipitated with the 2C2 mAb from an extract of amastigotes prepared by boiling the parasites in 1% SDS (lane 2).

However, if the parasites were lysed in NP-40 or Triton X-100 instead of boiling SDS, all of the [^3H]myristic acid label was lost, and Ssp-4 changed from hydrophobic to hydrophilic (Table I), similarly to what has been observed with VSG when *T. brucei* was subjected to analogous treatments. The removal of the COOH-terminal diacylglycerol from VSG is mediated by an endogenous phospholipase C and reveals the glycan-associated CRD epitope (2, 3). To determine whether a similar glycan was present in Ssp-4, amastigotes of *T. cruzi* were surface labeled with ^{125}I , lysed with NP-40, and samples of the extracts were immunoprecipitated with either mAb 2C2 or antibodies to CRD. The immunoprecipitated material was then subjected to 2D-electrophoresis and autoradiography. We found that the CRD epitope was present both in the 70,000 and the 84,000 M_r bands of Ssp-4. Moreover, although the 2D gels revealed additional heterogeneity of Ssp-4, identical patterns were seen in either immunoprecipitated material (Fig. 4).

FIGURE 2. Immunogold labeling of Ssp-4 on amastigotes of *T. cruzi* after 6 (*top*) and 48 h (*bottom*) of extracellular incubation. The arrows point to unlabeled trypomastigotes present in the same preparation. Bars, 1 μm .

TABLE I
Triton X-114 Partition of Extracts of ^{125}I Surface-labeled Amastigotes
and of the Culture Medium Obtained after a 48-h Incubation of the
Parasites at 37°C

Material subjected to partition	cpm* associated with SSP-4	
	Aqueous phase	Detergent phase
Triton X-100 lysates	1,203	27
Boiling SDS lysates	112	1,924
Culture medium	2,641	261

* Minus background obtained by immunoprecipitation with unrelated mAb.

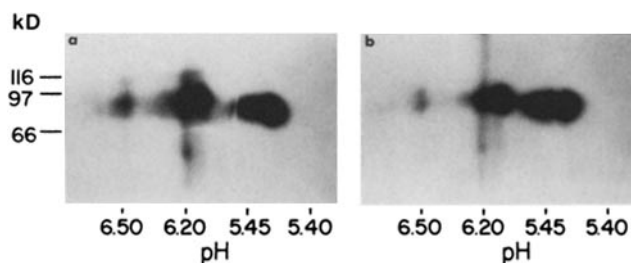


FIGURE 4. Two-dimensional electrophoretic analysis of Ssp-4. Amastigotes of *T. cruzi* were surface labeled with ^{125}I and extracted in NP-40. Shown are two-dimensional patterns resulting from immunoprecipitation with the (a) 2C2 mAb or (b) anti-CRD antibodies followed by autoradiography.

The similarity between the Ssp-4 and VSG GPI anchors was further supported by the observation that the hydrophobic Ssp-4 present in the boiled SDS extracts of amastigotes was converted into the hydrophilic, CRD-containing form by treatment with a purified preparation of phospholipase C from *T. brucei* (16) (not shown).

Ssp-4 is Released from the Membrane during Development. Having established that Ssp-4 is GPI anchored, next we studied its expression during development. Ssp-4 was found on the surface of amastigotes in relatively high amounts during the first hours of intracellular life (Fig. 5a) and throughout the first division cycles (Fig. 5b). At ~72 h after infection of cells, a diffuse material reacting with the mAb was detected in their cytoplasm (Fig. 5c), while the intensity of the parasite-associated fluorescence decreased (Fig. 5d).

Similarly, the amounts of Ssp-4 decreased with time on amastigotes originating from incubation at 37°C of trypomastigotes in medium, in the absence of mammalian cells. By FACS analysis, we found that about 70% of surface membrane-associated Ssp-4 was lost during 72 h in culture (Fig. 6, see legend). After 48 h of in vitro development, the amounts of Ssp-4 detected by immunogold labeling on the surface of amastigotes was substantially decreased, but the distribution remained uniform (Fig. 2, bottom). As assessed by fluorescein diacetate fluorescence, the viability of the parasites during the whole incubation period was >95%.

Mechanism of Release of Ssp-4. To study the properties of released Ssp-4, purified amastigotes, obtained by incubating trypomastigotes at 37°C in the absence of mammalian cells, were surface labeled with ^{125}I . The parasites were washed and further incubated in the culture medium at 37°C. At various times samples of SDS-boiled parasites and the corresponding supernatants were im-

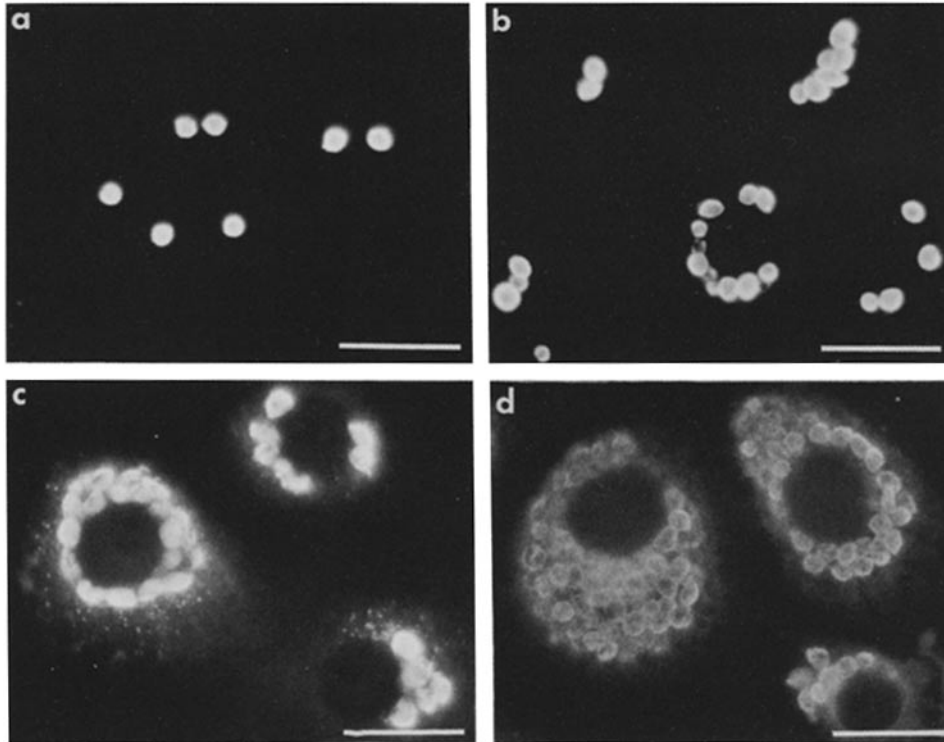


FIGURE 5. Intracellular expression of Ssp-4 during *T. cruzi* development. LLC-MK₂ cells fixed with methanol (*a*) 8, (*b*) 48, (*c*) 72, and (*d*) 96 h after infection with trypomastigotes were subjected to indirect immunofluorescence staining with the 2C2 mAbs. Photomicrographs were taken with the same exposure time and printed identically. Bars, 10 μ m.

munoprecipitated either with mAb 2C2 or with antibodies to CRD. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Fig. 7 shows Ssp-4 recovered by immunoprecipitation of SDS-boiled cell extracts (lanes 1–4) and the corresponding incubation medium (lanes 5–8) with saturating amounts of either the 2C2 mAb (Fig. 7A) or anti-CRD (Fig. 7B). Clearly, only Ssp-4 found in the culture medium was reactive with anti-CRD antibodies. We calculated that ~50% of the ¹²⁵I-labeled Ssp-4 originally associated with the surface of the cells was detected in the supernatant after 48 h. Between 10 and 48 h in culture, >90% of the released Ssp-4 was recoverable by immunoprecipitation with excess anti-CRD antibodies. Immunoprecipitation with mAb 2C2 was somewhat less efficient (Fig. 7C).

Next we studied the properties of the ¹²⁵I-labeled Ssp-4 which was shed and found that >90% of it behaves as a hydrophilic protein when partitioned in Triton X-114 (Table I). In contrast, only 5% of Ssp-4 in boiled SDS extracts of parasites is hydrophilic. The conversion of Ssp-4 from hydrophobic to hydrophilic form was associated with loss of lipid, as shown in a separate experiment in which amastigotes were metabolically labeled with both [³⁵S]methionine and [³H]myristic acid. While Ssp-4 containing both labels could be immunoprecipitated from

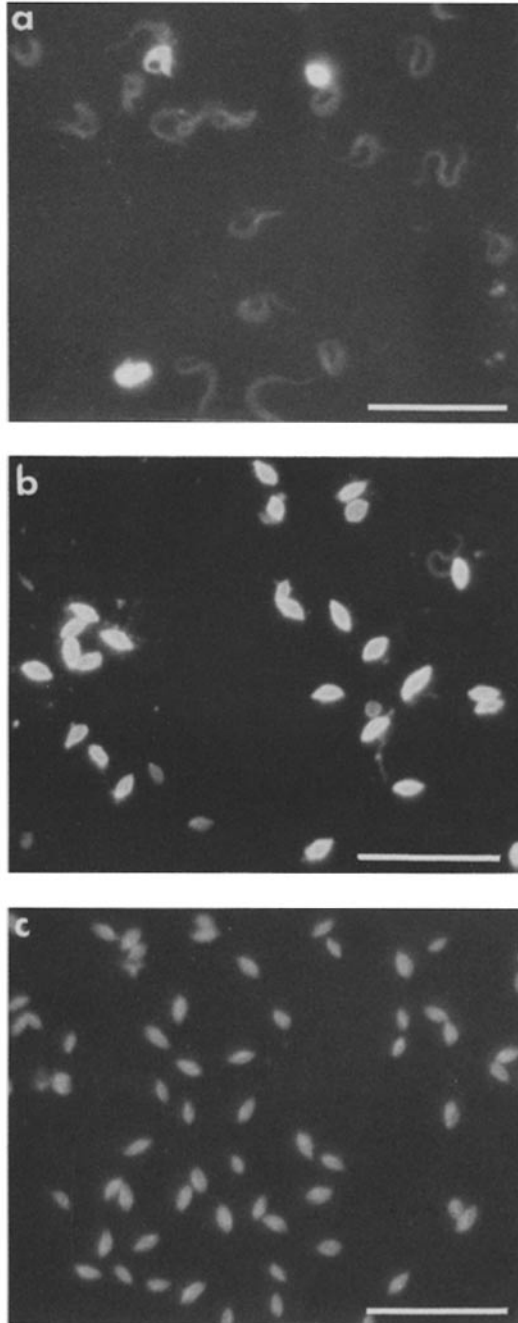


FIGURE 6. Surface expression of Ssp-4 during extracellular development of *T. cruzi*. Indirect immunofluorescence assays were performed with the mAb 2C2 on parasites fixed after different periods of incubation at 37°C in the absence of mammalian cells. (a) 0; (b) 24; (c) 72 h. The mean fluorescence intensity, measured by FACS analysis, was (a) 245, (b) 157, (c) 82. All photomicrographs were taken with the same exposure time and printed identically. Bars, 10 µm.

boiled SDS lysates of parasites, the molecule present in the incubation medium was deficient in labeled myristic acid (Table II).

These experiments demonstrated that all of the released Ssp-4 had the properties expected from the activity of a phospholipase C on the membrane form of

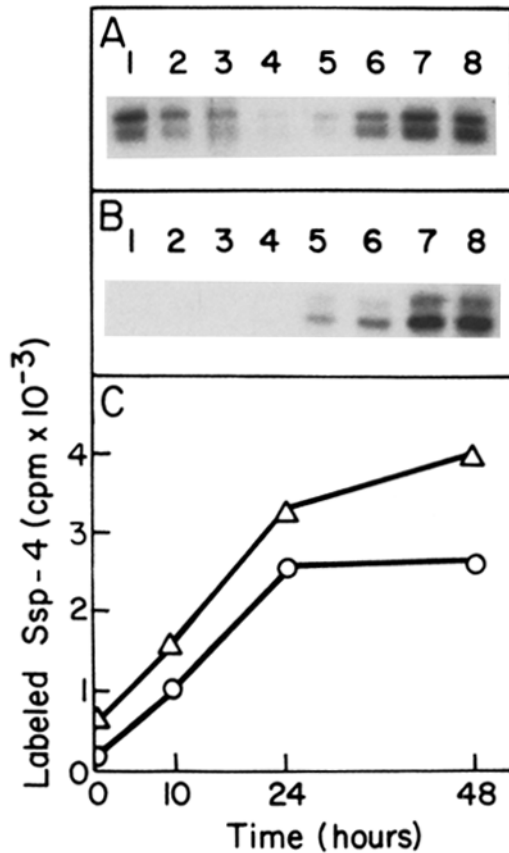


FIGURE 7. Kinetics of Ssp-4 release from the membrane of amastigotes of *T. cruzi*. (A) SDS-PAGE analysis of extracts of ¹²⁵I surface-labeled amastigotes (lanes 1–4) and samples of the corresponding culture medium (lanes 5–8) after immunoprecipitation with mAb 2C2; (B) same, after immunoprecipitation with anti-CRD antibodies; (C) total cpm immunoprecipitated from the culture medium by the 2C2 mAb (○) or anti-CRD (Δ) at each time point. Lanes 1 and 5, 0 h of incubation; 2 and 6, 10 h; 3 and 7, 24 h; 4 and 8, 48 h.

TABLE II
Presence of [³⁵S]Methionine and [³H]Myristic Acid Label in Ssp-4 from Cell Extracts or Ssp-4 Released into the Incubation Medium

Incorporated molecules	cpm* immunoprecipitated from	
	Boiled SDS extracts of parasites	Incubation medium
[³⁵ S]Methionine	1,918	3,816
[³ H]Myristic acid	1,322	178

* Minus background obtained by immunoprecipitation with unrelated mAb.

the molecule. Additional studies revealed the presence of phospholipase in extracts of different developmental stages of *T. cruzi* (Table III). The parasites were lysed in 1% NP-40 and extracts from each stage incubated separately with purified [³H]myristate-labeled VSG from *T. brucei* at 37°C. The membrane form of VSG was hydrolyzed in all samples and a product of the reaction was identified by TLC as [³H]dimyristoylglycerol.

The phospholipase C from *T. brucei* (16–18) and the activity from *T. cruzi* shared several properties; they were active in the absence of divalent cations and

TABLE III
Hydrolysis of [³H]Myristate-labeled VSG by Extracts of *Trypanosoma Cruzi*

Parasite extracts (in 1% NP-40)	Protein* con- centration	Inhibitors	<i>n</i> -Butanol soluble cpm [‡]
	μ g		
Amastigotes	10.0	—	1,386
	5.0	—	755
	2.5	—	528
	10.0	cm ^{ps} [§]	355
	10.0	protease inhibitors [¶]	1,401
Trypomastigotes	10.0	—	1,374
	5.0	—	876
	2.5	—	580
Epimastigotes	10.0	—	180
	5.0	—	121
	2.5	—	78
Positive control <i>T. brucei</i> GPIPLC (7.5 U) [†]			1,589

* Determined by BCA protein assay reagent (Pierce Chemical Co.).

‡ Minus background.

§ 5 mM *p*-chloromercuriphenylsulfonic acid.

¶ 1 mM PMSF and *N*- α -tosyl-L-lysine-chloromethyl ketone; 10 μ g/ml aprotinin and leupeptin.

† Phospholipase C purified from *T. brucei*.

could be partially inhibited by *p*-chloromercuriphenylsulfonic acid, but not by the protease inhibitors PMSF, *N*- α -tosyl-L-lysine-chloromethyl ketone, leupeptin, and aprotinin (Table III). Enzymatic activity was destroyed by boiling extracts of the parasite in SDS.

Discussion

While many membrane proteins from mammalian cells are anchored by means of a short stretch of hydrophobic amino acids, recent evidence indicates that some are attached by a glycosylated phosphatidylinositol moiety. The functional significance of this unusual anchor is unknown, but it facilitates lateral mobility of the proteins (19, 20) and renders possible their removal by phospholipases. The latter is supported by the finding that in protozoan parasites, which frequently and rapidly change their surfaces during development, several characterized membrane proteins are anchored by GPI (1, 2).

In African trypanosomes, VSG, which is linked to the membrane via GPI, is lost from the surface of the parasite during its transformation into the promastigote stage in the insect vector. Although the parasite contains a GPI-specific phospholipase C, the direct involvement of the enzyme in the shedding process has been difficult to ascertain in the complex *in vitro* system necessary for the bloodstream–promastigote transformation (21). In this system, parasite viability is a major cause for concern, because the phospholipase C becomes active upon cell lysis. Nonetheless, prior studies suggest a role for the endogenous phospholipase C in this transformation, although most of the released VSG is in the form of a proteolytic fragment, rather than soluble VSG (22, 23).

The above difficulties were not encountered in the *T. cruzi* model. This report

demonstrates that the major surface protein of amastigotes of *T. cruzi* (Ssp-4) is GPI anchored and that it is released during their differentiation in vitro extra- or intracellularly. In both situations the shedding of Ssp-4 precedes the transformation of the parasite into epimastigotes, which are transient forms intracellularly, but constitute an important replicative form in culture or in the insect host. The present studies show that the parasite contains an endogenous phospholipase C and most or all Ssp-4 shed in the culture medium has the properties expected from a fragment generated through the activity of such an enzyme.

Ssp-4 is an acidic, mannose-containing 70,000–84,000 M_r glycoprotein, which homogeneously covers the whole surface of recently transformed amastigotes in a manner suggestive of a densely packed coat. When parasites are kept in vitro, under conditions in which they are fully viable, Ssp-4 is shed. After 48 h of incubation, ~50% of the ^{125}I surface-labeled Ssp-4 molecules can be recovered from the culture medium. The shed Ssp-4 differs from the membrane form and its properties are indistinguishable from the product generated by hydrolysis of the isolated membrane form of Ssp-4 by purified GPI-specific phospholipase C: it does not contain myristic acid, it partitions in Triton X-114 as a hydrophilic molecule, and most or all of it reacts with antibodies to CRD.

The latter constitutes compelling evidence that the cleavage was mediated by the endogenous phospholipase C. Antibodies to CRD are directed against epitopes present at the COOH-terminal end of soluble *T. brucei* VSG (24), and are only exposed after removal of diacylglycerol by phospholipase C. The CRD determinant is shared by several other GPI-anchored proteins (25–28), and is critically dependent on the presence of the terminal inositol-phosphate. Although the glycan moiety participates in the epitope (29), the presence of the phosphate group is essential for antibody recognition of CRD as shown by competitive inhibition experiments with simple sugars. For example, D-L myo-inositol 1,2 cyclic monophosphate significantly inhibited antibody reactivity with GRD at concentrations of between 10^{-5} and 10^{-4} mM, while myoinositol had no effect at a concentration of 5 mM (Shak, S., M. A. Davitz, M. L. Wolinsky, V. Nussenzweig, M. J. Turner, and A. Gurnett, submitted for publication). In addition the antibodies to CRD did not recognize the product generated by the cleavage of a GPI-anchored molecule by phospholipase D (30; Cardoso de Almeida, M. L., B. U. Stambuck, M. J. Turner, and S. Schenkman, submitted for publication).

The finding that Ssp-4 is recognized by the glycan-specific *T. brucei* phospholipase C and that the *T. cruzi* enzyme released diacylglycerol from the membrane form of VSG argues that the glycans of Ssp-4 and VSG are similar. The *T. cruzi* and the *T. brucei* enzymes in fact share several properties; they are Ca^{++} independent, inhibited by *p*-chloromercuriphenylsulfonic acid, destroyed by boiling in SDS, but remain active in parasite extracts prepared in non-ionic detergents.

One of the most intriguing problems still unsolved is the mechanism leading to the gradual release of Ssp-4 as *T. cruzi* develops. In the case of *T. brucei*, the biosynthesis of VSG stops very soon after a shift in culture conditions, but the shedding process is also slow and proceeds for several hours afterwards (22). In contrast, if either *T. brucei* or *T. cruzi* is lysed in NP-40, the cleavage of the GPI-anchored molecules is almost instantaneous, indicating that the enzyme is fully

active. These seemingly contradictory observations could be reconciled if the enzyme did not have access to the substrate in vivo. For example, it is conceivable that the phospholipase C is associated with a defined region of the parasite surface such as the flagellar pocket (31), and that cleavage only takes place when the substrate enters it, presumably during its migration within the lipid bilayer. Reconstitution experiments in phospholipid vesicles have shown that the *T. brucei* enzyme acts on VSG when both are present within the same phospholipid bilayer (17). Clearly, more information is needed about the cellular distribution of the phospholipase C in *T. cruzi* and how its activity is regulated.

The number of proteins which appear to be GPI-anchored has been growing steadily and includes functionally diverse molecules from the surface of eukaryotic cells. Several are found in circulation in vivo or released from intact cells in culture (2). It is possible that they are not secreted in the classical sense, but that enzymatic activities similar to those described here mediate their removal from the surface membrane. It is interesting to note that if either a phospholipase C or D is involved, cleavage of the protein would liberate, within the lipid bilayer, molecules that could potentially act as second messengers, such as diacylglycerol or phosphatidic acid (32, 33). Clearly, in the case of parasites, these coordinated events are ideally suited to integrate structural membrane changes with signals for differentiation. For example, *T. cruzi* continuously changes in the mammalian host from trypomastigote to amastigote regardless of whether it enters cells or not, and during this transformation, membrane molecules Ssp-1, Ssp-2, Ssp-3, and Ssp-4 are sequentially expressed and lost (11). It would be of interest to determine whether, in addition to Ssp-4, the other membrane-associated stage-specific antigens are GPI-anchored, and whether the profound morphological changes are driven by signals initiated by anchor products.

Summary

The surface of amastigotes of *Trypanosoma cruzi* is covered by Ssp-4, a major stage-specific glycoprotein. Ssp-4 is anchored to the cell membrane by GPI. It can be metabolically labeled with [³H]myristic acid, and is converted into a hydrophilic form by treatment with the glycan-specific phospholipase C of *T. brucei*, or after lysis of the parasites in non-ionic detergents. The hydrophilic form of Ssp-4 is recognized by antibodies to the cross-reactive determinant of the variant surface glycoprotein of African trypanosomes.

Ssp-4 is progressively shed during the intra- or extracellular development of amastigotes preceding their transformation into epi- and trypomastigotes. We show here that *T. cruzi* contains a phospholipase C and that most shed Ssp-4 is hydrophilic, does not contain myristic acid, and reacts with anti-CRD. These observations provide strong evidence that phospholipase C mediates the release of this glycosyl-phosphatidylinositol-anchored protein under physiological conditions, as the parasite undergoes differentiation.

We are grateful to Drs. Dale Hereld and Paul Englund for their gift of purified *T. brucei* phospholipase C, and to Dr. David Russell for supplying anti-CRD antibodies. We thank Ms. Monica Einstein for excellent technical assistance, Ms. Iwona Gumper for EM specimen preparation, and Mr. Roger Rose for help in preparing the manuscript.

Received for publication 29 September 1987.

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