Articles

Variant Surface Glycoprotein from *Trypanosoma evansi* Is Partially Responsible for the Cross-Reaction between *Trypanosoma evansi* and *Trypanosoma vivax*†

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ABSTRACT: Salivarian trypanosomes use antigenic variation of their variant-specific surface glycoprotein (VSG) coat as a defense against the host immune system. Although about 1000 VSG and pseudo-VSG genes are scattered throughout the trypanosome genome, each trypanosome expresses only one VSG, while the rest of the genes are transcriptionally silent. A 64-kDa glycosylated cross-reacting antigen between *Trypanosoma evansi* and *Trypanosoma vivax* (p64), which was purified from the TEVA1 *T. evansi* Venezuelan isolate, was proven here to represent the soluble form of a VSG. Initially, a biochemical characterization of p64 was carried out. Gel filtration chromatography, sedimentation, and chemical cross-linking provided evidences of the dimeric nature of p64. The hydrodynamic parameters indicated that p64 is asymmetrical with a frictional ratio $f/f_o = 1.57$. Isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis revealed that p64 contained two isoforms with isoelectric points of 6.8−6.9 and 7.1−7.2. When p64 and three p64 *Staphylococcus aureus* V8 proteolytic fragments were sequenced, the same N-termini sequence was obtained: Ala-Pro-Ile-Thr-Asp-Ala-Asp-Leu-Gly-Pro-Ala-Gln-Ile-Ala-Asp, which displayed a significant homology with a putative *Trypanosoma brucei* VSG gene located on chromosome 4. Additionally, immunofluorescence microscopy on *T. evansi* and *T. vivax* established that p64 and its *T. vivax* homologue were confined to the surface of both parasites. An immunological characterization of this antigen was also carried out using several Venezuelan *T. evansi* isolates expressing different VSGs, which were obtained from naturally infected animals. Although sera from animals infected with the various *T. evansi* isolates recognized p64, only one isolate, besides TEVA1, contained polypeptides that were recognized by anti-p64 antibodies. All these results together with prior evidences [Uzcanga, G. et al. (2002) *Parasitology 124*, 287−299] confirmed that p64 is the soluble form of a *T. evansi* VSG, containing common epitopes recognized by sera from animals infected with *T. evansi* or *T. vivax*. Despite the huge repertoire of VSG genes existing on bloodstream trypanosomes, our data also demonstrated the potential use of a VSG variant from the TEVA1 *T. evansi* isolate as a diagnostic reagent.

The Salivarian trypanosomes are intravascular, extracellular haemoprotozoan parasites of a wide range of mammals including humans. Although trypanosomes appear initially in the blood, they also move into the extravascular spaces of deeper tissues and frequently invade the central nervous system. On the basis of their morphology and development in the vector, they have been subdivided into four subgenera: *Trypanozoon*, *Duttonella*, *Nannomonas*, and *Pycnomonas* (*1*). In Venezuela, two nontsetse transmitted trypanosomes, *Trypanosoma* (*Trypanozoon*) *evansi* and *Trypanosoma* (*Duttonella*) *vivax*, are the major etiological agents of animal trypanosomosis and generate a significant economic impact in livestock. *T. vivax* affects predominantly bovines while *T. evansi* causes the disease known as derrengadera in equines. Infections are associated with progressive weakness, intermittent fever, anemia, weight loss, nervous signs, and eventually death. Both *T. evansi* and *T. vivax* are mechanically transmitted by biting insects including *Tabanus*, *Cryptotylus*, and *Stomoxys* species (*2*). Additionally, vampire bats (*Desmodus rotundus)*, ticks, and triatomine bugs have also been suggested as vectors in the transmission of *T. vivax*.

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Like all Salivarian trypanosomes, *T. vivax and T. evansi* evade the immune system of the host using an antigenic variation strategy (*3*, *4*). These trypanosomes live in the blood and lack intracellular stages, so the parasite is a target for antibody-mediated destruction. Certainly, almost all trypanosomes are cleared from blood by liver macrophages (*5*, *6*). However, the remaining parasites survive and establish the infection thanks to the antigenic variation of the so-called variant surface glycoprotein (VSG).[1] Each trypanosome carries a large repertoire of VSG genes coding for multiple VSG variants with a different primary sequence, particularly at the N-termini (*7*−*9*). Approximately 1000 VSG and pseudo-VSG genes are distributed throughout the trypanosome genome, all of which are transcriptionally silent except for one (*10*−*12*). Each parasite expresses a single VSG gene at any one time by replacing the previous gene at the telomeric active site of transcriptional expression with the new one (*13*). In consequence, this repeated antigenic change of VSG in trypanosomes allows them to evade the thymus-dependent humoral response, resulting in successive surges of parasitaemia, a situation similar to being infected successively by related, but not identical, pathogens. This phenomenon makes it difficult to develop a vaccine against the disease. Additionally, the dense surface coat formed by the closely packed monolayer of VSGs is essential for the parasite life in the host's bloodstream, preventing complement-mediated lysis and providing a macromolecular barrier that hinders host molecules from interacting with the parasite's plasma membrane.

T. evansi and *T. vivax* have shown a very high immunological cross-reactivity, which has been previously reported using capillary agglutination (*14*), ELISA (*15*, *16*), indirect immunofluorescence, and Western blot assays (*17*). The production of *T. vivax* antigens is a limiting factor in most laboratories (*18*). The host restriction of most stocks of *T. vivax* to ruminants and the relatively low level parasitaemias in their natural host have combined to make *T. vivax* an unpopular subject for experimental studies (*19*). On the contrary, rodents can be experimentally infected with *T. evansi* to obtain enough parasites to prepare *T. evansi* antigens for serological tests. We have recently identified antigens responsible for the cross-reactivity between *T. evansi* and *T. vivax*, and the predominant 64-kDa antigen, p64, was purified from the TEVA1 *T. evansi* isolate (*17*). On the basis of its relative abundance, its probable location on the parasite surface, its release into the parasite soluble fraction following extractions, the presence of carbohydrate and the glycosylphosphatidylinositol (GPI) moiety on its structure, and its apparent molecular weight by SDS−PAGE, we suggested that p64 corresponded to the soluble form of a VSG variant from this *T. evansi* isolate (*17*). In this paper, we evaluated various biochemical parameters and immunochemical properties of p64 to confirm our initial idea.

[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CRD, cross-reacting determinant; DAB, diaminobenzidine; GPI, glycosylphosphatidylinositol; NBT, nitro blue tetrazolium; p64, a 64-kDa antigen from *T. evansi* that exhibits cross-reactivity with *T. vivax*; o-PDM, *N*-*N*′-1,2 phenylenedimaleimide; p-PDM, *N*-*N*′-1,4 phenylenedimaleimide; PMSF, phenyl methyl sulfonyl fluoride; poly(ethylene glycol) compound, 2,2′-[(methyl-ethylidene)-bis (4,1-phenyleneoxymethylene)]-bis-oxirane polymer with α-hydro-*ω*-hydroxypoly(oxy-1,2-ethanediyl]; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; sulfo-SMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; VSG, variant surface glycoprotein; sVSG, soluble form of VSG.

EXPERIMENTAL PROCEDURES

Materials. Reagents were purchased from the following sources: middle range molecular weight protein markers, prestained protein ladder, anti-mouse IgG horseradish peroxidase conjugate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), Promega; anti-bovine IgG alkaline phosphatase conjugate, anti-equine IgG alkaline phosphatase conjugate, fluorescein-conjugated anti-bovine IgG, benzamidine, iodoacetamide, phenyl methyl sulfonyl fluoride (PMSF), fibrous DEAE-cellulose, diaminobenzidine (DAB), *N*-*N*′-1,2 phenylenedimaleimide (o-PDM), *N*-*N*′-1,4 phenylenedimaleimide (p-PDM), gel filtration molecular weight protein marker kit, *Staphylococcus aureus* V8 protease, 2,2′-[(methyl-ethylidene)-bis (4,1-phenyleneoxymethylene)]-bis-oxirane polymer with α-hydro-*ω*-hydroxypoly(oxy-1,2-ethanediyl] (poly(ethylene glycol) compound), methyl-α-D-mannopyranoside, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), Sigma; Q-Sepharose, S-Sepharose, Sephacryl S-300, broad range isoelectric focusing calibration kit (pH 3−10), Immobilin dry strips (pH 3−10), ampholites (pH 3−10), ConA-Sepharose, Pharmacia; and fluorescein-conjugated anti-mouse IgG, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), and succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), Pierce. All other chemicals were of the highest quality grade available.

Parasites. Eight Venezuelan *T. evansi* isolates were employed including the TEVA1 isolate previously characterized by Desquesnes and Tresse using PCR (*18*). The other seven Venezuelan isolates were acquired from naturally infected horses, donkeys, and capybaras: isolates TeAp-Mantecal and TeGu-Cab were obtained from horses, TeGub-323 and TeGub-trino were separated from infected donkeys, and TeAp-Cedral 05, TeAp-Cedral 12, and TeAp-El Frío were attained from capybaras. PCR reactions were used for amplification of a 960-bp minicircle DNA that flanked the *T. evansi* minicircle conserved region. All these isolates were identified as *T. evansi* (data not shown) since the sequences of the generated products were highly homologous to the reported sequences for minicircles from African and Asian *T. evansi* parasites (*20*, *21*).

T. evansi parasites were expanded into adult albino rats (Sprague Dawley). When the number of parasites reached 10^6−10^8 trypanosomes/mL, the blood was extracted from the rats by cardiac puncture using EDTA as an anticoagulant. Trypanosomes were purified by anion-exchange chromatography using fibrous DEAE-cellulose (*22*). Parasites eluting from the column were collected by centrifugation at 1475*g*, for 20 min at 4 °C, and washed three times with 20 mM phosphate buffer (pH 7.2) containing 1% glucose. *T. evansi* parasites were counted by a hemocytometer, and the final cell pellet was kept frozen at −70 °C until further use.

A cryopreserved sample of *T. vivax* (∼10^6 parasites), originally isolated from a naturally infected bovine from the Falcon State, Venezuela, was expanded by inoculating it into a goat. Goat infected blood was cryopreserved and subsequently used for further experimental infections of healthy

bovines. This trypanosome was identified as *T. vivax* since the cryopreserved parasites did not expand into adult albino rats. Additionally, these parasites presented a distinctive motion in wet blood films, consisting of a rapid vibrational movement often followed by speedy translational movement across the field of view (*23*). The concentration of *T. vivax* parasites was determined using a Neubauer chamber.

Source of Antigens. T. evansi parasites were homogenized on ice by sonication, using 2 mL of buffer A [5 mM Tris-HCl (pH 7.2), 1 mM benzamidine, 1 mM PMSF, 5 mM EDTA, 1 mM iodoacetamide]. In some experiments, the *T. evansi* whole-cell homogenate was used as the source of antigens. In other cases, the parasite extract was incubated for 5 min at room temperature, and then it was centrifuged at 15 000*g* for 30 min at 4 °C to obtain the supernatant and pellet fractions. The pellet fraction was reextracted once more, incubated for 5 min at room temperature, and centrifuged as stated previously. Both resulting supernatants were collected and employed as the source of parasite antigens and were defined as the clarified antigenic fraction from *T. evansi*.

Purification of p64. Cryopreserved *T. evansi* parasites from the Venezuelan TEVA1 isolate were used to purify the p64 antigen. The clarified antigenic fraction originating from 9.6×10^9 *T. evansi* parasites homogenized with 40 mL of buffer A was loaded onto a Q-Sepharose column (50 mL) connected in tandem with a S-Sepharose column (10 mL) (*17*). Under these conditions, p64 eluted in the nonadhering fraction. Alternatively, p64 was purified by affinity chromatography throughout ConA-Sepharose. In this case, the resin was equilibrated in buffer B [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$]. The clarified antigenic fraction from 5×10^9 parasites was incubated with 3 mL of ConA-Sepharose at 4 °C, for 2 h, with gentle agitation. The flow-through material was recollected by low-speed centrifugation, and the resin was washed with buffer B containing 0.5 M NaCl, until the absorbance at 280 nm was approximately zero. Incubating the resin with buffer B in the presence of 0.5 M methyl-α-D-mannopyranoside eluted p64.

Zymograms. The proteolytic activity present in the fractions from the ConA-Sepharose chromatography was evaluated by zymograms, using electrophoresis in gelatin-containing sodium dodecyl sulfate (SDS)−polyacrylamide slab gels (*24*).

Protein Determination. Protein concentration was determined as reported by Bradford (*25*), using BSA as protein standard.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed on 1.5 mm thick slabs containing 12 or 15% polyacrylamide (*26*). Coomassie blue R-250 or silver staining was used for protein visualization.

Western Blot Analyses. Proteins separated by SDS−PAGE were electrotransferred from the gels to nitrocellulose sheets (0.45 μm pore size) according to the procedure described by Towbin et al. (*27*). Sera of naturally infected and parasitologically positive animals were withdrawn and employed for immunodetection: asn 323 and asn-trino obtained from infected donkeys, cab-4 separated from an infected horse, chig-05 and chig-119 attained from infected capybaras, and buf-453 acquired from an infected buffalo. The nitrocellulose filters were incubated with animal sera (dilution 1:100) or specific anti-p64 polyclonal antibodies (dilution 1:10 000) (*17*). Then, the antigenically reacting proteins were incubated with the appropriate dilution of alkaline phosphatase conjugated or horseradish peroxidase conjugated secondary antibodies against equine, bovine, or mouse IgG depending on the case, following the instructions of the supplier. On the basis of evolutionary relationships, secondary antibodies against equine, bovine, and mouse IgG were respectively used in the cases when sera from donkeys, buffalo, and capybaras were employed as the primary antibodies. Finally, the protein bands were visualized by the addition of NBT and BCIP when alkaline phosphatase-conjugated antibodies were used, or DAB and hydrogen peroxide when horseradish peroxidase-conjugated antibodies were employed, according to the provider.

Gel Filtration Chromatography. Following concentration by dialysis at 4 °C against the solid poly(ethylene glycol) compound, p64 (1 mg) was applied to a Sephacryl S-300 size-exclusion column [total volume (V_t) = 50.3 mL] previously equilibrated with 50 mM Tris (pH 8), 150 mM NaCl, and 5 mM β-mercaptoethanol. Protein standards were used to calibrate the column. The excluded (V_o) and included volumes were determined by chromatographing blue dextran and potassium dichromate, respectively. The column was run at a flow rate of 150 μL/min, and the eluting proteins were monitored at 280 nm and subsequently separated by SDS−PAGE. The elution volume (V_e) was measured for each protein, and the corresponding K_{av} was calculated from eq 1.

$$K_{av} = (V_e - V_o)/(V_t - V_o) \quad (1)$$

The molecular weight of p64 was empirically determined by plotting the K_{av} value of each standard versus the logarithm of its molecular weight. Additionally, a linear relationship was obtained by plotting $(-\log K_{av})^{1/2}$ against each Stokes radius (*28*).

Sucrose Gradient Centrifugation. Linear 10−30% sucrose gradients (4.2 mL) were prepared in 100 mM Tris-HCl (pH 8), 0.2 mM EDTA, 0.4 mM dithiothreitol, 10 mM $MgCl_2$, and 0.3 M NH_4Cl. Standard proteins with known sedimentation coefficients were used to calibrate the gradients (*29*). Samples containing p64 (150 μg) and all protein markers (150 μg), in a total volume of 300 μL, were layered on top of various sucrose gradients. The samples were spun for 18 h, at 200 000*g*, in a Beckman SW 60Ti rotor, at 4 °C. Fractions were collected from the bottom of the tubes, and aliquots were analyzed by SDS−PAGE. The migration of p64 was determined by direct protein staining and Western blot analysis using anti-p64 polyclonal antibodies. In addition, the volume of migration of each standard was plotted against its corresponding sedimentation coefficient, and the resulting linear curve was utilized to calculate the sedimentation coefficient of p64 (*30*). The molecular mass of p64 was estimated from a calibration curve of the protein standards molecular masses versus their Stokes radii multiplied by their sedimentation coefficients.

Cross-Linking of p64. Samples of p64 were incubated for 1 h at room temperature, with several cross-linking reagents: SMCC (2 mM), Sulfo-SMCC (2 mM), o-PDM (1.5 mM), and p-PDM (1.5 mM). Stock solutions of SMCC, o-PDM, and p-PDM were freshly prepared in dimethylformamide,

and Sulfo-SMCC was dissolved in water. The reactions with SMCC and Sulfo-SMCC were carried out in 10 mM sodium phosphate (pH 7.2) and 5 mM magnesium acetate, while the reactions with o-PDM and p-PDM were performed in 25 mM Tris (pH 7.5) and 2.5 mM magnesium acetate. As controls, p64 was incubated with just the corresponding buffers in the presence or absence of dimethylformamide. The samples were separated by SDS−PAGE, and the cross-linked products were stained with Coomassie blue. Additionally, parallel gels were electrotransferred to nitrocellulose and revealed with polyclonal anti-p64 antibodies as described previously.

Isoelectric Focusing of p64. The isoelectric point of p64 was evaluated using a Mini Rotofor Cell (Bio-Rad). A preparative sample of p64 (500 μg) was fractionated for 3 h, at 4 °C, at a constant power of 12 W, throughout an ampholyte gradient (pH 3−10). Following the focusing step, 20 fractions were harvested, and their pH values were determined. Fractions were analyzed by SDS−PAGE, and the gels were colored with Coomassie blue.

Two-Dimensional Electrophoresis Analysis of p64. A sample of p64 (21 μg) was heat-denatured in the presence of 2% β-mercaptoethanol. The sample was precipitated with cold 80% acetone and dissolved in 2% ampholytes (pH 3−10), 9 M urea, 30 mM β-mercaptoethanol, and 0.5% Triton X-100. Isoelectric focusing was performed in a Multiphor system (Pharmacia), using an Immobilin polyacrylamide strip (13 cm) containing a linear ampholyte gradient (pH 3−10). Following the initial fractionation obtained in the first dimension, the strip was incubated with 350 mM Tris (pH 6.8), 6 M urea, 30% glycerol, 1.7% SDS, 5.4 mM dithiothreitol, and 0.02% bromophenol blue, for 10 min at room temperature, and placed on top of a 1 mm thick slab gel containing 12% polyacrylamide. The second dimension was performed as described by Laemmli (*26*), and the gel was stained with Coomassie blue.

Protein Sequencing. A sample of p64 (12.5 μg) was digested with 0.02 units of *S. aureus* V8 protease in 50 mM Tris-HCl (pH 8.1), following the procedure described by Down et al. (*31*). After incubation for 4.5 h, the reaction was stopped by heating the sample at 100 °C, for 5 min, with sample buffer for SDS−PAGE (*26*). The resulting proteolytic products were separated by SDS−PAGE on 15% polyacrylamide and electroblotted to polyvinylidene fluoride membranes (Immobilon-P, Millipore) in 10 mM CAPS (pH 11), 10% methanol (HPLC grade), and 0.1 mM sodium thioglycolate, for 1 h at 250 mA. p64 and three p64 *S. aureus* V8 proteolytic fragments were subjected to sequencing according to Matsudaira (*32*), using an automated protein microsequencer Prosize 493 cLC (PE-Applied Biosystems, Foster City, CA).

Indirect Immunofluorescence. The blood of rats infected with the TEVA1 isolate of *T. evansi* or of a *T. vivax*-infected bovine was extracted, and the cells were washed twice with PBS* [20 mM phosphate buffer (pH 7.2), 136.9 mM NaCl, 2.7 mM KCl] by centrifugation at 1475*g* for 20 min. The cells were resuspended in one volume of 2% bovine serum albumin (BSA) in PBS*, and aliquots of 10 μL were smeared on microscope slides. Immunofluorescence was carried out following the procedure described by Cons and Kaplan (*33*). Microscope slides precoated with blood containing *T. evansi* or *T. vivax* parasites were incubated with polyclonal antibodies produced in mouse ascitic fluid against the purified p64 from *T. evansi* (dilution 1:40) (*17*). These antibodies were prepared according to the procedure described by Bubis et al. (*34*). Then, a fluorescein-conjugated secondary antibody against mouse IgG (dilution 1:50) was employed. The parasites were viewed using a fluorescence microscopy directly connected with a photographic camera.

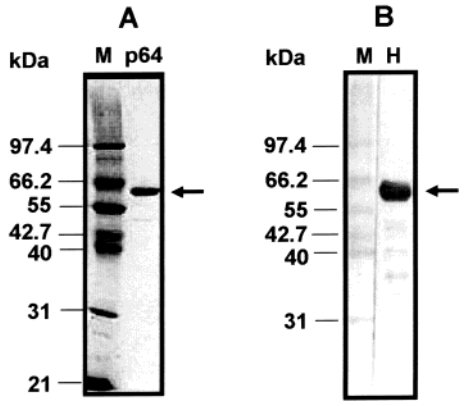

FIGURE 1: (A) Purified p64 was separated by SDS−PAGE and stained with Coomassie blue. (B) Recognition of p64 in whole *T. evansi* homogenates (H) by anti-p64 antibodies. M = protein markers.

Indirect ELISA. Indirect ELISA was carried out according to the method described by Aray et al. (*15*). Briefly, ELISA plates were sensitized with the purified p64 or the clarified antigenic fraction from *T. evansi* (160 ng of protein/well) in carbonate-bicarbonate buffer (pH 9.6), overnight at 4 °C, in a humid chamber. Then, blocking buffer [0.02 M sodium phosphate (pH 7.2), 0.15 M NaCl, 0.1% Tween 20, 5% skim milk] was applied in excess to each well for 1 h at 37 °C. An aliquot (100 μL) of equine, bovine, capybara, buffalo, or donkey serum from experimentally or naturally infected animals (1:100 dilution) was added per well. After an extensive wash, a horseradish peroxidase-conjugated secondary antibody against the corresponding IgG (dilution 1:2000) was supplemented using 100 μL/well. The color reaction was developed employing 10 mg of ABTS in 100 mL of 0.05 M phosphate-citrate buffer (pH 5) containing 0.0075% hydrogen peroxide. Sera from 32 naturally infected bovines living in a protozoan endemic area at the Venezuelan savanna were also evaluated. As negative controls, sera from 15 racehorses living in a trypanosomosis-free area at La Rinconada Racetrack, Venezuela, and from 30 healthy bovines living in France, which is a trypanosomosis nonendemic area, were also evaluated.

RESULTS

Purification of p64 from T. evansi. In comparison to our previous paper (*17*), the yield of p64 was highly increased when reextractions as well as 5 min resting periods, at room temperature, were included prior to each centrifugation step during the parasite homogenization procedure. With this modification, 10 mg of p64 was obtained starting from 9.6 $\times$ 10^9 parasites, which represented approximately 24% of the total parasite protein. This result confirmed that p64 corresponded to a major component of *T. evansi*. The purified p64 is shown in Figure 1A. Polyclonal anti-p64 antibodies prepared in mice ascitic fluid (*17*) specifically recognized p64 in whole *T. evansi* homogenates (Figure 1B).

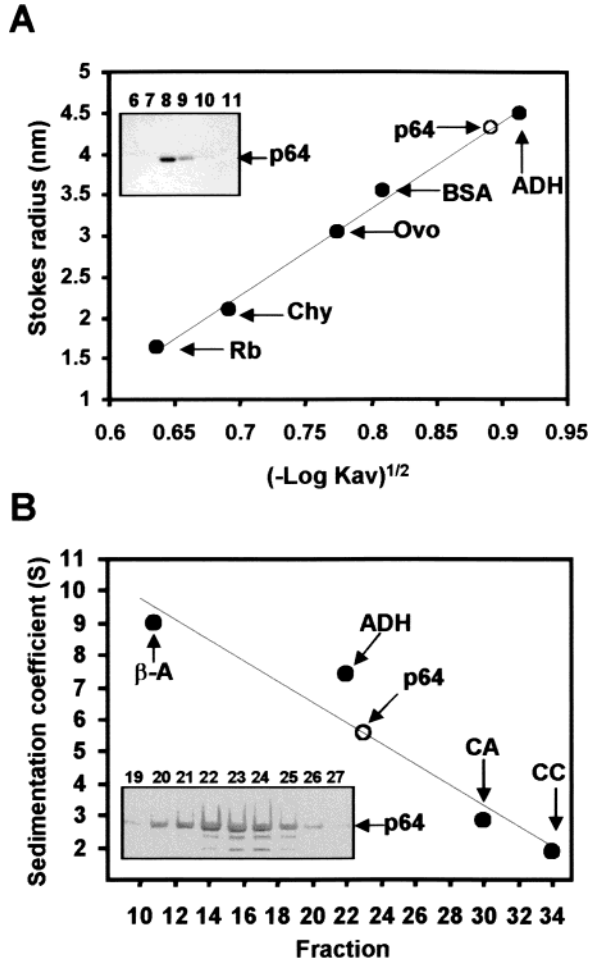

FIGURE 2: Determination of the hydrodynamic parameters of p64. (A) Gel filtration. The calibration curve for the Sephacryl S-300 column was established with alcohol dehydrogenase (ADH, 150 kDa, 45 Å), bovine serum albumin (BSA, 67 kDa, 35.5 Å), ovalbumin (Ovo, 43 kDa, 30.5 Å), chymotrypsinogen A (Chy, 25 kDa, 20.9 Å), and ribonuclease A (Rb, 13.7 kDa, 16.4 Å). The elution position of p64 (○) was determined by SDS−PAGE (inset). p64 eluted between column fractions 8 and 10, showing its major peak at fraction 8. (B) Sucrose gradient centrifugation. Gradient calibration was carried out with β-amylase (β-A, 8.89 S), alcohol dehydrogenase (ADH, 7.4 S), carbonic anhydrase (CA, 2.8 S), and cytochrome C (CC, 1.86 S) (*25*). The sedimentation position of p64 (○) was identified by Western blot using anti-p64 antibodies (inset). p64 sedimented between fractions 19 and 27, peaking at fraction 23.

Approximately 0.5−1 mg of p64 was achieved starting from 10^{11} parasites, when affinity chromatography throughout ConA-Sepharose was used (data not shown). As demonstrated by SDS−PAGE, no contamination with other proteins was observed following elution with 0.5 M methyl-α-D-mannopyranoside (data not included). Additionally, zymograms showed the absence of contaminating proteases in the fraction containing the ConA-Sepharose purified p64 (data not shown).

Gel Filtration Chromatography and Sucrose Gradient Sedimentation. The native molecular weight of p64 was estimated to be 122 000 by Sephacryl S-300 molecular exclusion chromatography (data not shown). The measured elution volume yielded the $(-\log K_{av})^{1/2}$ for p64, and a Stokes radius of 43.13 Å was determined by interpolation (Figure 2A). These results demonstrated that p64 corresponds to a dimer.

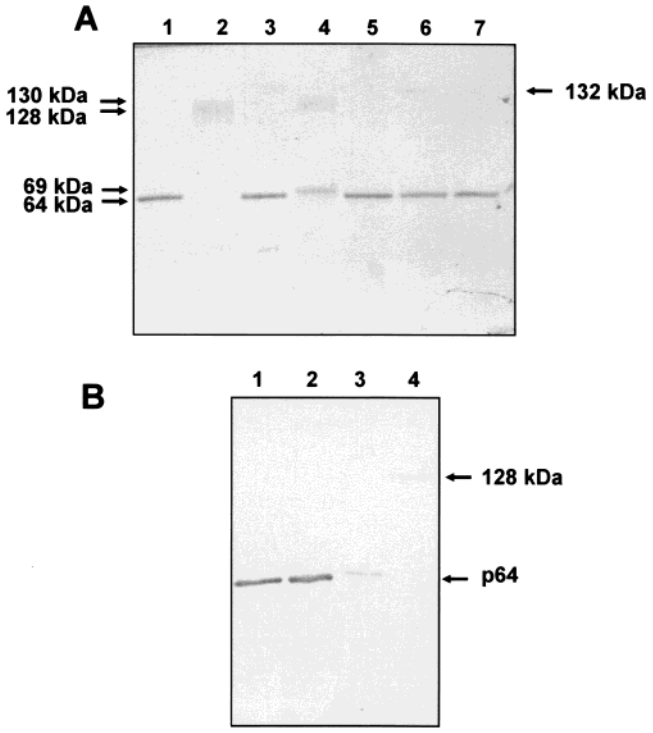

FIGURE 3: Incubation of p64 with various bifunctional agents. (A) p64 was incubated with Sulfo-SMCC (2), SMCC (4), o-PDM (6), or p-PDM (7). The various samples were separated by SDS−PAGE and stained with Coomassie blue. Lanes 1, 3, and 5 = control experiments containing p64 with the corresponding vehicles for the reactions with Sulfo-SMCC, SMCC, and both phenylenedimaleimides, respectively. (B) Samples of SMCC-treated p64 (3) and Sulfo-SMCC-treated p64 (4) were electrotransferred to nitrocellulose and revealed with anti-p64 antibodies. Lanes 1 and 2 = control experiments for the reactions with Sulfo-SMCC and SMCC, respectively.

Velocity sedimentation experiments showed that the sedimentation coefficient for p64 was 5.67 S (Figure 2B). For spherical molecules, the molecular mass of a species can be calculated from a combination of the measured Stokes radius and sedimentation coefficient using eq 2 (*28*, *35*)

$$M = 6\pi\eta Nas(1 - \nu\rho) \quad (2)$$

in which M is the molecular mass, a is the Stokes radius, s is the sedimentation coefficient, ν is the partial specific volume, η is the viscosity of the medium, ρ is the density of the medium, and N is Avogadro's number. A calibration curve of M versus the Stokes radius multiplied by the sedimentation coefficient was prepared using the values reported for the protein markers, and a molecular mass of 105 000 was estimated for p64 (data not included). This size was consistent with p64 being dimeric in structure.

The frictional coefficient f/f_o can also be determined from the molecular mass and the Stokes radius as seen in eq 3.

$$f/f_o = a/(3\upsilon M/4\pi N)^{1/3} \quad (3)$$

The calculated frictional ratio for p64 was 1.57. This f/f_o value suggested some asymmetry in the protein molecule and indicated that the native p64 dimer folds into a conformation that lies in the boundary between globular and moderately elongated (*36*).

Covalent Cross-Linking of p64. Figure 3 resumes the results of incubating p64 with several cross-linking reagents.

As expected, the migration of p64 on SDS−PAGE was not modified in the control samples (Figure 3A, lanes 1, 3, and 5). On the other hand, p64 was cross-linked to various degrees depending on the bifunctional compound employed. Sulfo-SMCC and SMCC are reagents capable of forming bridges between Cys and Lys spatially located about 11.6 Å apart. Sulfo-SMCC stoichiometrically cross-linked p64 (Figure 3A, lane 2), producing a diffuse polypeptide band that migrated with an apparent molecular mass of 128 kDa. The incubation of p64 with SMCC partially yielded a slightly slower-migrating cross-linked band of about 130 kDa (lane 4), but the reaction did not undergo completion. Additionally, the treatment with SMCC changed the migration of the original p64 band to an apparent molecular weight of 69 000 (lane 4), which suggested the modification of the protein by one of the chemical functions of the heterobifunctional reagent, without the formation of cross-linked products. Interestingly, polyclonal anti-p64 antibodies did not recognize the 130-kDa polypeptide band obtained by incubating p64 with SMCC (Figure 3B, lane 3) but slightly recognized the 128-kDa band cross-linked by Sulfo-SMCC (Figure 3B, lane 4). o-PDM and p-PDM are specific-homobifunctional agents that cross-link Cys residues located at 9 or 12 Å, respectively (*37*). Although p-PDM did not cross-link p64, o-PDM was capable of cross-linking p64, yielding a minor amount of a diffuse band with an apparent molecular weigh of 132 000 (Figure 3A, lanes 6 and 7). In all cases, the molecular weight range observed was consistent with the formation of dimeric cross-linked products, which again evidenced that native p64 is a dimer.

64-kDa Polypeptide Band Consists of Two Isoforms. Two spots (a and b) were revealed by two-dimensional electrophoresis separation of p64 (Figure 4A, inset). Using protein markers, the calculated isoelectric points of a and b were 6.9 and 7.1, respectively (Figure 4A). In addition, two peaks for p64 were obtained at pH 6.8 and 7.2 when p64 was separated by isoelectric focusing on a Rotofor chamber (Figure 4B). These electrofocused peaks must correspond to the a and b isoforms of p64, respectively. Both methods evidenced that p64 contained two isoforms with isoelectric point values close to 7.

Partial Sequence of p64. p64 and three polypeptides obtained by its proteolysis with the *S. aureus* V8 protease were subjected to N-terminal amino acid sequencing. The first 15 amino acid residues of p64 and of the three polypeptides were identical, corresponding to the following sequence: Ala-Pro-Ile-Thr-Asp-Ala-Asp-Leu-Gly-Pro-Ala-Gln-Ile-Ala-Asp. According to the emerging *Trypanosoma brucei* genome project databases (http://tigrblast.tigr.org/er-blast/index.cgi?project=tba1), this sequence displayed a significant homology (73% identities, 80% similarities) with a putative *T. brucei* VSG gene located on chromosome 4. This result evidenced that p64 must correspond to a *T. evansi* VSG.

Immunolabeling of T. evansi and T. vivax with Anti-p64 Antibodies. Anti-p64 polyclonal antibodies heavily labeled whole *T. evansi* (Figure 5B) and *T. vivax* (Figure 5D) parasites, including the flagella. No labeling was obtained on the cell nucleus in either case. No fluorescence was observed when fluorescein-conjugated anti mouse IgG was employed alone, or when another ascitic fluid produced against the γ-subunit of bovine transducin, a nonrelated protein, was used (Figure 5A,C).

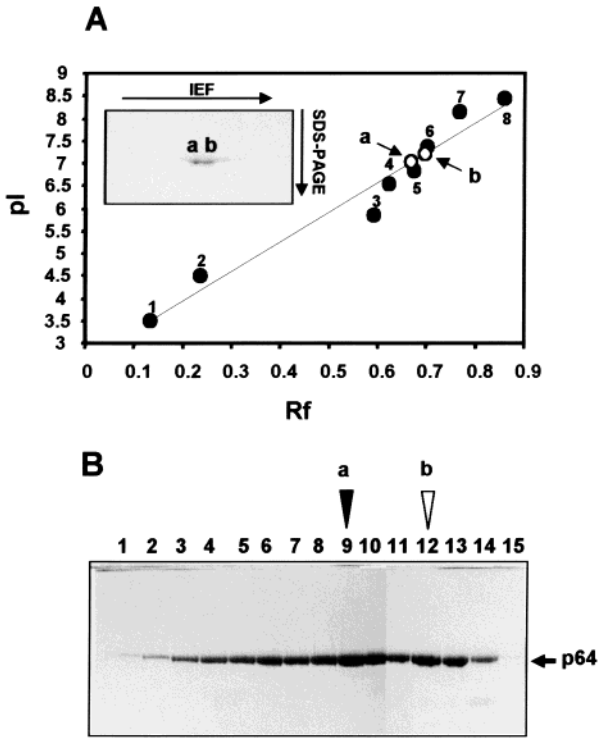

FIGURE 4: Purified p64 contained two isoforms. (A) Two-dimensional electrophoresis separation of purified p64 (inset). The pIs of both p64 isoforms, a and b (○), were empirically determined plotting the pI value of each standard vs the relative mobility (R_f). The following protein markers were used to establish the pI calibration curve: amylglucosidase (1, pI 3.5), soy trypsin inhibitor (2, pI 4.55), β-lactoglobulin A (3, pI 5.2), bovine carbonic anhydrase B (4, pI 5.85), human carbonic anhydrase B (5, pI 6.55), acidic band of horse myoglobin (6, pI 6.85), basic band of horse myoglobin (7, pI 7.35), acidic band of lentil lectin (8, pI 8.15), and middle band of lentil lectin (9, pI 8.45). (B) Purified p64 was separated by electrofocusing on a Mini-Rotofor chamber. Two p64 peaks were obtained at fractions 9 and 12 (arrowheads).

Membrane-Bound p64 Has an Apparent Molecular Weight of 61 000 by SDS−PAGE. Soluble p64 has an apparent molecular mass 3000 Da higher than p64 present on trypanosomes directly lysed by boiling with SDS−PAGE sample buffer (Figure 6A, lanes 1 and 2, respectively). As shown in Figure 6B, anti-p64 antibodies did not recognize the intact p64 (Figure 6B, lane 2).

Anti p64 Antibodies Only Recognized Antigenic Bands on Homogenates of Two T. evansi Isolates. The protein profile of the extracts from eight different *T. evansi* isolates is shown in Figure 7A. A predominant polypeptide band of apparent molecular mass between 40 and 65 kDa is observed in all samples, which may represent their corresponding VSGs. As previously reported, anti-p64 antibodies immunorecognized p64 in the TEVA1 *T. evansi* isolate (Figure 7B, lane 4). Additionally, anti-p64 reacted with two bands in the TeGu-Cab *T. evansi* isolate, a 64 and a 52 kDa polypeptide (Figure 7B, lane 3). None of the other putative VSGs were recognized by the anti-p64 antibodies (Figure 7B). When the separation was performed under nonreducing conditions, anti-p64 recognized a polypeptide band of about 109 kDa in the TeGu-Cab *T. evansi* sample (Figure 7C, lane 3). This result indicated the formation of disulfide bonds between

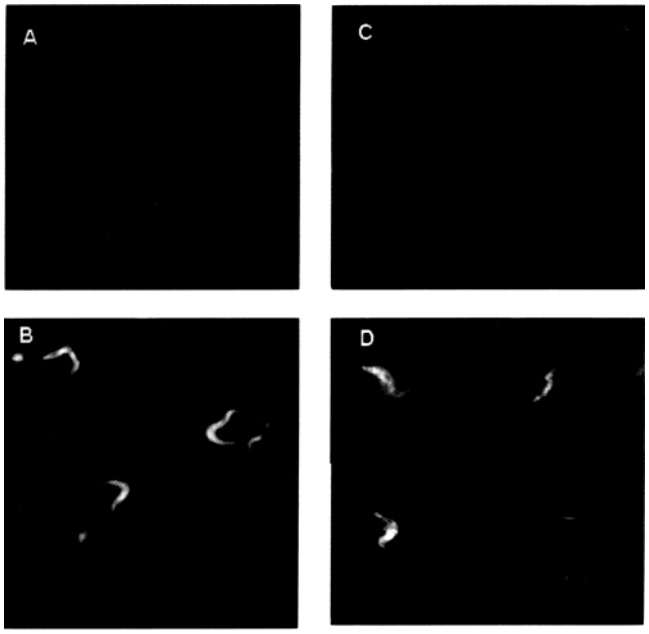

FIGURE 5: Immunolabeling of *T. evansi* and *T. vivax* with anti-p64 antibodies. Microscope slide glasses, precoated with blood containing *T. evansi* (B) or *T. vivax* (D) parasites, were incubated with anti-p64 antibodies. (A) Sections containing *T. evansi* parasites were incubated with fluorescein-conjugated anti-mouse IgG without pretreating the sections with anti-p64 antibodies. (C) *T. vivax* parasites were incubated with antibodies against a nonrelated protein, the γ-subunit of bovine transducin. Magnification = 800×.

the corresponding monomers. In contrast, no difference was observed when the TEVA1 sample was run in the absence of β-mercaptoethanol (Figure 7C, lane 4), indicating that p64 and the polypeptide bands that bind to anti-p64 in the TeGu-Cab *T. evansi* isolate have different primary structures. Other high-molecular mass polypeptide bands (>200 kDa) were also detected in all parasite extracts (Figure 7C). However, identical bands were revealed when a nonrelated ascitic fluid produced against the γ-subunit of visual transducin was employed (data not shown), which suggested a nonspecific recognition.

All Sera Obtained from Parasitological Positive Animals Immunorecognized p64. Naturally and experimentally infected animals were examined for the presence of trypanosomes in the circulation by the micro-haematocrit technique (*38*). Sixteen animals (five bovines, five horses, three donkeys, two capybaras, and one buffalo) resulted in being positive by this method. Interestingly, the sera from all these parasitological positive animals immunorecognized p64 by indirect ELISA. Additionally, Western blot analyses illustrated that p64 was also recognized by sera from infected animals with six different *T. evansi* isolates, some of which were employed in Figure 7 (Figure 8, lanes 1−6). No polypeptide bands were detected when the corresponding secondary antibodies were used alone as controls (Figure 8, lanes 7−9).

Detection of Anti-p64 Antibodies in the Sera of Naturally Infected Bovines by Indirect ELISA. Sera from 32 bovines, which were highly seropositive by indirect ELISA using the clarified fraction from *T. evansi*, were selected to monitor for the immunorecognition of the purified p64. Indirect ELISA showed that 78% of the evaluated sera contained anti-p64 antibodies.

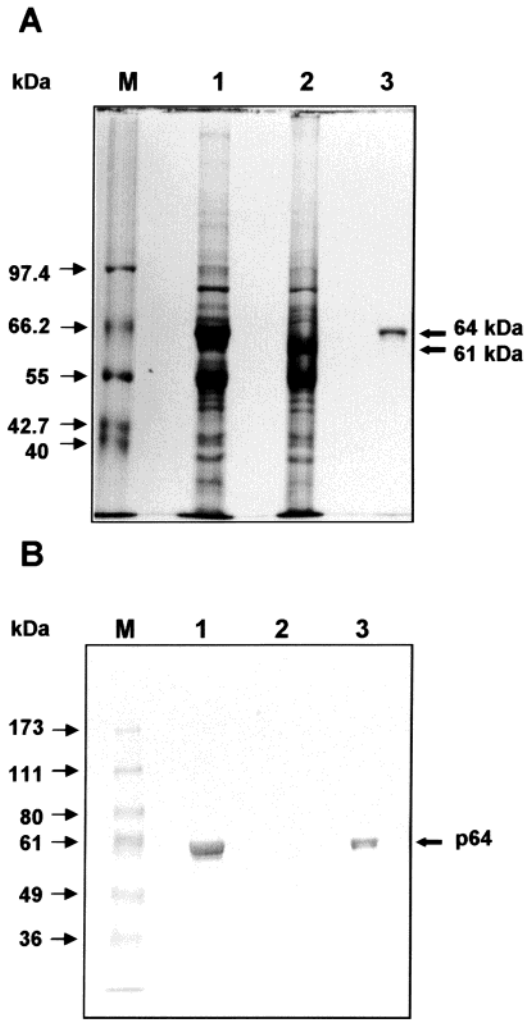

FIGURE 6: Comparison between soluble p64 and membrane-bound p64. *T. evansi* parasites were homogenized by the protocol described in the Experimental Procedures (1) or directly lysed by boiling with SDS−PAGE sample buffer (2). Purified p64 was included in lane 3. M = protein markers. (A) SDS−PAGE separation visualized by Coomassie blue staining. (B) Western blot analyses using anti-p64 antibodies.

DISCUSSION

Salivarian trypanosomes escape antibody-mediated destruction through periodic changes of the expressed VSG gene from a repertoire of approximately 1000 (*12*). They keep one step ahead of the immune system by continually switching from the expression of one VSG on their surface to the expression of another immunologically distinct VSG, a phenomenon called antigenic variation (*10*−*12*). Accordingly, it has been generally accepted that a particular VSG variant could not be considered as a potential antigen for diagnostic purposes and less as a cross-reacting antigen between trypanosome species. However, our results have demonstrated that despite the huge repertoire of VSG genes existing on these trypanosomes, equine anti-*T. evansi* and bovine anti-*T. vivax* antibodies from experimentally infected animals immunorecognized the soluble form of a VSG purified from the TEVA1 isolate of *T. evansi*. Moreover, sera obtained from naturally and experimentally infected animals also bind to this particular VSG variant. The phylogenetic analysis, based on bootstrapped maximum

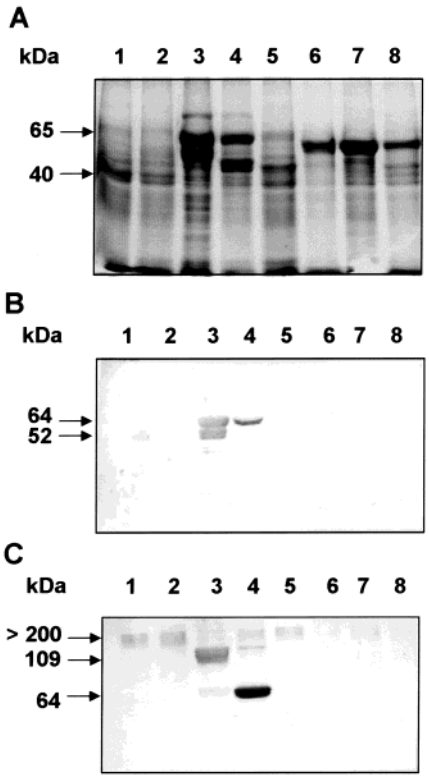

FIGURE 7: (A) Whole-cell extracts from eight Venezuelan *T. evansi* isolates were separated by SDS−PAGE and analyzed by Coomassie blue staining. Identical samples prepared under reducing (B) or nonreducing conditions (C) were evaluated by Western blot using anti-p64 antibodies. Gel lanes contained the following samples: TeGub-323 (1), TeGub-trino (2), TeGu-Cab (3), TEVA1 (4), TeAp-Mantecal (5), TeAp-El Frío (6), TeAp-Cedral 12 (7), and TeAp-Cedral 05 (8).

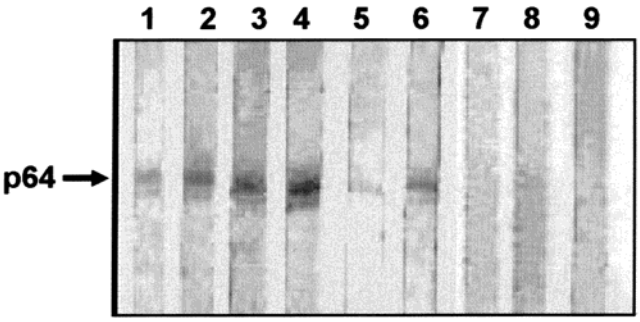

FIGURE 8: Reactivity of p64 with sera from naturally infected animals. Blots containing p64 were cut into 4 mm strips. Strips were developed using the following sera: asn-trino (1) and asn 323 (2), which were obtained from infected donkeys; cab-4 (3) separated from an infected horse; buf-453 (4) acquired from an infected buffalo; and chig-05 (5) and chig-119 (6) attained from infected capybaras. Strips 7−9 were directly incubated with secondary antibodies against equine, bovine, and mouse IgG, respectively, as controls.

parsimony analysis of the small subunit (18S) rRNA gene, places the Salivarian trypanosomes in a monophyletic clade, the *T. brucei* clade, comprising exclusively mammalian trypanosomes of African origin: *T. brucei*, *Trypanosoma congolense*, *Trypanosoma simiae*, *Trypanosoma godfreyi*, and *T. vivax* (*39*, *40*). Additionally, *T. evansi* and *Trypanosoma equiperdum* also belong to this clade by virtue of their close morphological and genetic similarity to *T. brucei*. Analyses of kinetoplast DNA (*41*) and isoenzymes (*42*, *43*) point to *T. evansi* and *T. equiperdum* being comparatively recent derivatives of *T. brucei*, which have been able to spread outside Africa because they no longer rely on tsetse transmission. Similarly, *T. vivax* has been imported into South America in the recent past (*44*). On the basis of the tree published by Stevens and Gibson (*39*), *T. vivax* is positioned on the edge of the group, as a sister clade of the subclade represented by *T. brucei*, *T. evansi*, and *T. equiperdum*. It is very significant to find immunological cross-reactivity between VSG variants from species belonging to sister clades. Moreover, this result establishes the potential use of a particular *T. evansi* VSG variant as a diagnostic reagent for both equine and bovine trypanosomosis.

Since hypotonic lysis causes the release of an endogenous phospholipase C-like activity in *T. brucei* that cleaves the glycolipid anchor and releases the parasite VSG in a soluble form (*45*, *46*), we introduced changes in the purification protocol of p64 to promote this enzymatic activity in *T. evansi*. As a result, we achieved a 17% yield increase in p64. Ten milligrams of p64 was obtained from 9.6×10^9 parasites, which approximated typical VSG yields from *T. brucei* that have ranged from 30 to 60 mg per 10^{11} cells (*46*). The p64 antigen was also purified to homogeneity by ConA-Sepharose chromatography, resulting in a very low yield (0.5−1 mg of purified p64 from 10^{11} parasites), which agreed with results reported by Boutignon et al. (*47*) for the *T. brucei* VSG. Although by definition VSG molecules are very heterogeneous in terms of their amino acid sequence and both their glycosylation pattern and number of glycosylation sites, they all share one conserved N-linked $GlcNAc_2$-Man_{5-9} moiety (*48*). Since p64 is the soluble form of a *T. evansi* VSG, this triantennary mannose *N*-glycan structure must be responsible for the tight binding of p64 to the lectin resin. As a result, only a minor fraction of p64 was purified from this column, probably corresponding to incompletely glycosylated proteins. The final yield obtained by ConA-Sepharose chromatography unequivocally indicates that this is probably a step to avoid in the purification of p64.

T. brucei VSGs are dimers associated through hydrophobic interactions and in some cases covalently bound via disulfide bridges (*47*, *49*). A disulfide-linked VSG dimer was also reported in a *T. evansi* clone (MIAG.065) (*50*). Our results obtained by molecular exclusion chromatography demonstrated that p64 has a molecular weight of 122 000, approximately twice the apparent molecular weight of 64 000, observed by SDS−PAGE under denaturing conditions. A Stokes radius of 43.13 Å for p64 was also determined, which again indicated the dimeric structure of the protein. Gurnett et al. (*51*) compared the solution properties of membrane and soluble forms of *T. brucei* VSGs and obtained a sedimentation coefficient of 5.5 S and a frictional ratio of 1.22 for the soluble form. These values are in reasonable agreement with our results for p64 that showed a sedimentation coefficient of 5.67 S and a frictional ratio of 1.57. By assuming a globular and compact shape for p64, a molecular weight of 105 000 was estimated for the protein. However, most biological macromolecules are not spheres, and ellipsoids of revolution (prolate or oblate ellipsoids) are more realistic models than a sphere. Ellipsoids have larger frictional coefficients than equivalent spheres. Because the volume of a molecule is proportional to the molecular weight, it has been reported that the more a molecule deviates from

a sphere, the larger its frictional coefficient will become. The f/f_o value determined for p64 corresponds to a protein having either a prolate ellipsoid shape with an axial ratio of 10:1 or an oblate ellipsoid shape with an axial ratio of 12:1 (*52*).

Freyman et al. (*53*) determined the 2.9 Å resolution structure of the N-terminal domain of a VSG from *T. brucei*. The crystal structure showed that the N-terminal domain molecule is a dimer, ~100 Å long. The molecule has a strikingly asymmetric cross-section, being ~60 × 40 Å at the bottom, ~30 × 20 Å in the middle, and ~45 × 45 Å near the top (*53*). These results are consistent with the dimeric structure and asymmetric shape reported for the *T. brucei* VSG, as well as with the similar properties observed here for the *T. evansi* p64.

Purified p64 was also shown to exist in solution as a dimer as judged by SDS−PAGE after treatment with bifunctional cross-linking agents. Both Sulfo-SMCC and SMCC cross-linked p64, indicating the presence of vicinal Cys and Lys residues located within a distance of about 11.6 Å. However, only Sulfo-SMCC was capable of carrying out the reaction stoichiometrically. Since Sulfo-SMCC is a polar compound, the cross-linked amino acids seemed to be located in hydrophilic regions of the protein. o-PDM and p-PDM, two homobifunctional reagents used to cross-link Cys residues located approximately 9 or 12 Å apart, respectively, were not as efficient as Sulfo-SMCC and SMCC. p-PDM did not cross-link p64, and o-PDM was capable of cross-linking only a minor amount of p64. Apparently, the distances between the reactive vicinal thiol groups of p64 are not adequate for the formation of cross-linked linkages with o-PDM and p-PDM. Additionally, SDS−PAGE analyses demonstrated that disulfide bridges neither exist in the native p64 nor spontaneously form when the protein was denatured under nonreducing conditions (Figure 7C, lane 4). Clearly, disulfide linkages are not implicated in the maintenance of the p64 dimeric structure. Moreover, these results are consistent with the cross-linking experiments using phenylenedimaleimides, which suggested either the absence of vicinal intermolecular sulfhydryl groups or the existence of only a few neighboring thiol groups between the subunits forming the dimer. Interestingly, some *T. brucei* VSGs have also been reported to exist as dimers by cross-linking studies (*49*, *51*).

Since p64 did not bind to either Q-Sepharose or S-Sepharose, and eluted in the flow-through fraction during the purification procedure, the isoelectric point of the protein can be roughly estimated to be near neutrality. Although p64 migrated as a single band on SDS−PAGE, two isoforms were found after isoelectric focusing, with pI values of 6.8−6.9 and 7.1−7.2, respectively. These values agreed with the pIs reported for various *T. brucei* VSGs variants, which appeared heterogeneous after isoelectric focusing (*54*, *55*).

Cardoso de Almeida and Turner (*56*) demonstrated a molecular weight change associated with the release of soluble VSGs from *T. brucei*. Similarly, soluble p64 has a higher apparent molecular weight than p64 present on trypanosomes that have been lysed by boiling with SDS−PAGE sample buffer. This result, together with immunoblot analyses using antibodies directed against the cross-reacting determinant (CRD) of the hydrophilic gp63 from *Leishmania mexicana* (*57*), revealed the presence of a GPI membrane anchor on p64 (*17*). Interestingly, intact p64 was not detected by Western blot using polyclonal anti-p64 antibodies, which initially suggested that the CRD moiety was the immunological determinant recognized by the antibodies. However, Uzcanga et al. (*17*) have shown that anti-p64 did not recognize the CRD moiety of the GPI anchor described by Zamze et al. (*58*) since mild acid treatment did not destroy the epitope. Likewise, Bütikofer et al. (*59*) have found that the removal of lipid moieties from the anchor can have an effect on the antigenicity of a variety of GPI-anchored surface molecules. Several antibodies raised against GPI-anchored proteins were not longer capable of binding the corresponding antigens once the lipid moieties had been removed. Conversely, antibodies raised against soluble and delipidated forms reacted poorly with intact GPI-anchored proteins but showed enhanced binding after treatment with phospholipases (*59*). Barboni et al. (*60*) have suggested that the conformational changes that accompanied delipidation are probably responsible for the changes in antigenicity. One possible explanation is that lipids in the GPI anchor might affect the conformation of a protein by acting as a constraint at its carboxyl terminus. Immunofluorescence experiments revealed that anti-p64 antibodies were capable of binding to parasites that had been permeabilized by acetone fixation. However, this procedure destroys biological membranes and most likely causes the release of the parasite GPI-phospholipase C, resulting in the partial delipidation of the GPI anchor (*59*). On the other hand, we have also seen that anti-p64 antibodies bind to living trypanosomes using in vivo indirect immunofluorescence (data not shown). Therefore, the lack of recognition of the intact protein by the anti-p64 antibodies following immunoblotting must be probably related to hindrance associated with the presence of the GPI group or to artifacts linked to the transference of the protein to nitrocellulose membranes.

When various *T. evansi* isolates were evaluated by Western blotting, only two isolates, TEVA1 and TeGu-Cab, showed polypeptide bands immunorecognized by the anti-p64 antibodies. These results indicated that p64 was not a regular or invariable protein among isolates. The recognition of p64 in the TEVA1 isolate was anticipated because p64 was purified from it. However, p64 and the corresponding TeGu-Cab homologues do not correspond to the same proteins. In contrast to p64, which migrated as a monomer on nonreducing SDS−PAGE, the immunodetected polypeptides from the TeGu-Cab isolate migrated as a dimer when electrophoresed in the absence of β-mercaptoethanol, suggesting that these proteins possess different primary structures. Since our procedure releases the soluble form of the VSGs variants from the various *T. evansi* isolates and exposes their CRD moiety, evidently anti-p64 does not recognize this new epitope. Therefore, since different isolates probably express distinct VSGs molecules, our results confirmed that p64 must represent a TEVA1 VSG variant. We also demonstrated here that sera obtained from animals infected with the various *T. evansi* isolates recognized p64. Moreover, naturally infected bovines contained anti-p64 antibodies, and healthy bovines lacking anti-trypanosome and/or anti-p64 antibodies developed a humoral response against p64 following experimental infection with *T. vivax* (*17*). These results verified the presence of cross-reactivity epitopes among different VSG variants and reaffirmed that p64 corresponded to a cross-reacting antigen from *T. evansi*. Recently, Claes et al. (*61*) reported that *T. equiperdum*, a sexually transmitted parasite

that causes the disease known as dourine in horses, can express VSGs containing epitopes serologically similar to those on the VSG from an isolated *T. evansi* strain (RoTat 1.2). Previously, the RoTat 1.2 VSG was only proven to appear in *T. evansi* and not in other trypanosomes from the *Trypanozoon* subspecies, including *T. brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense* (*62*). The data of Claes et al. also indicated that a *T. evansi* VSG is a cross-reacting antigen (*61*), which agrees with our results. However, *T. equiperdum* is morphologically identical to *T. evansi*, and the authors reported that they could not exclude the possibility that positive *T. evansi* strains might have been misclassified as *T. equiperdum* stocks. Additionally, since both parasites occur together in many regions of the world, current diagnostic tests are consistently unable to distinguish between them. In our case, the various Venezuelan isolates used here were correctly classified as *T. evansi*. PCR reactions were employed to amplify a 960-bp minicircle DNA that flanked the *T. evansi* minicircle conserved region, and all generated products were highly homologous to the reported sequences for minicircles from African and Asian *T. evansi* parasites (*20*, *21*). These results, together with direct microscopic visualization using Giemsa staining (data not included), also demonstrated that all our *T. evansi* isolates were kinetoplastic and differed from dyskinetoplastic *T. evansi* stocks reported in other South American countries (*63*). Additionally, *T. equiperdum* trypanosomes are rarely observed in the bloodstream of the host because they are normally localized in the capillaries of the mucous membranes of the urogenital tract. Although few *T. equiperdum* parasites occasionally appear in the peripheral blood of animals with chronic infection, this is considered to be very rare (*64*). Finally, although *T. equiperdum* has been described in other South American countries, *T. equiperdum* has not yet been reported in Venezuela.

Anti-CRD antibodies labeling experiments, together with immunofluorescence microscopy using anti-p64 antibodies, demonstrated that VSG is located on the *T. evansi* surface. Unexpectedly, anti-p64 antibodies recognized a p53 kDa polypeptide band in whole-cell homogenates of purified *T. vivax* (*17*). When *T. vivax* was analyzed by indirect immunofluorescence, the parasite surface was also labeled. Thus, p53 must represent a VSG variant from the *T. vivax* isolate used here.

In *T. brucei*, the *S. aureus* V8 protease cleaves some VSG variants into an N-terminal domain and a C-terminal domain, containing 350−400 residues and 50−100 residues, respectively (*31*, *65*). Analysis of the sequences of *T. brucei* VSGs has revealed, with some exceptions, little conservation of primary sequence within the N-terminal domain (*7*, *66*, *67*). There is, however, substantial sequence conservation in the C-terminal, including four sets of four or eight cysteine residues, which has led to the assignment of VSGs to one of four classes (*68*). Despite low sequence similarity, there are also four partially conserved cysteine residues in the N-terminal part of the molecule. These have been shown to form two intramolecular disulfide bonds in some VSG variants (*69*). Sequencing of p64 and three p64 *S. aureus* V8 proteolytic fragments revealed the same sequence, which corresponded to the N-termini. Although the N-terminal domain is the more variable region among VSGs, the p64 N-termini displayed an important homology with a putative *T. brucei* VSG gene, according to the *T. brucei* genome project database (http://tigrblast.tigr.org/er-blast/index.cgi?project=tba1). Since p64 corresponds to a VSG, the protein sequence that was achieved here provided strong evidence that the designation of the nucleotide sequence as a putative *T. brucei* VSG gene was correct. Although two isoforms were found for p64 by isoelectric focusing, only one sequence was obtained. In view of the fact that the parasite population expresses predominantly one VSG gene during a parasitaemia peak, the heterogeneity obtained for p64 must probably result from differential posttranslational modifications.

ACKNOWLEDGMENT

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