Purification of a 64 kDa antigen from *Trypanosoma evansi* that exhibits cross-reactivity with *Trypanosoma vivax*

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SUMMARY

Trypanosoma evansi and *Trypanosoma vivax* are the most extensively distributed trypanosomes responsible for diseases in livestock. Western blot and indirect immunofluorescence assays revealed a high immunological cross-reaction between these two parasites. An antigen with an apparent molecular mass of 64 kDa (p64), which exhibited cross-reactivity with *T. vivax*, was purified to homogeneity from a Venezuelan isolate of *T. evansi*. This antigen is glycosylated, contains a glycosyl-phosphatidylinositol anchor and appeared to be localized through the cell except in the nucleus, indicating that it could primarily be confined to the parasite surface. These results, together with its relative abundance and apparent molecular weight, suggest that p64 probably corresponds to the soluble form of a variable surface glycoprotein from *T. evansi*. Anti-p64 polyclonal antibodies, raised on mice, recognized a 53 kDa polypeptide band from a Venezuelan isolate of *T. vivax* on Western blots. Additionally, sera obtained from naturally infected animals also recognized p64, suggesting its potential use as a diagnostic reagent. Mild acid treatment only slightly decreased the immunorecognition of p64, suggesting its potential use as a diagnostic reagent. Mild acid treatment only slightly decreased the immunorecognition of p64, demonstrating that another relevant cross-reacting epitope, different than the inositol-1,2-cyclic phosphate of the cross-reacting determinant, must exist in p64. To date, p64 represents the first antigen isolated and partially characterized from *T. evansi*.

Key words: *Trypanosoma evansi*, *Trypanosoma vivax*, cross-reactivity, antigen, protein purification, variable surface glycoprotein.

INTRODUCTION

Trypanosomes responsible for diseases in livestock and domestic animals belong to several species including *T. congolense*, *T. simiae*, *T. vivax*, *T. brucei*, *T. evansi*, and *T. equiperdum*. Among these, *T. vivax* and *T. evansi* are the most widely spread since they occur in tropical Africa, south-eastern Asia (Boyd & Mleche, 1985), and South America (Shaw & Lainson, 1972; Wells, 1984; Applewhaite, 1990). Trypanosomiasis caused by these 2 non-tsetse transmitted parasites, represents a potential and dangerous risk for 500 million cattle, 100 million buffaloes, and 12 million camels that live in these endemic areas (Peregrine, 1994). For example, in Brazilian Pantanal and Bolivian Lowlands, losses have been estimated to exceed 17% of the total brood cow value (Seidl, Dávila & Silva, 1999). Interestingly, more than 11 million head of cattle valued at more than 3 billion US$ are found in this area.

The trypanosomiasis by *T. evansi* or 'derrengadera' is considered one of the major causes of death in horses in Venezuela. A 69·3% prevalence of *T. evansi* in a sample of 140 horses from the Venezuelan savanna, has been determined using an indirect fluorescent antibody test (F. Garcia, personal communication). Toro *et al.* (1980) estimated a 21% prevalence of *T. vivax* in cattle nationwide. The disease syndromes associated with the infection of both *T. evansi* and *T. vivax* vary from chronic to acute and fatal, having the clinical symptoms of progressive weakness, emaciation, fever, anaemia, and death.

Non-polluting control methods using drugs against trypanosomes are currently used and can prove successful under certain conditions (Bauer *et al.* 1995). However, these methods require a thorough knowledge of the epidemiological situation, which is time and labour consuming. The classical parasitological examination by microscopy, using either the micro-haematocrit technique (Woo, 1970) or the buffy-coat method (Murray, Murray & McIntyre, 1977), lacks sensitivity, detecting around 1000 trypanosomes/ml. Although the polymerase chain reaction is a very sensitive technique for detecting trypanosomes, in either the vector or the host (Moser *et al.* 1989; Masiga *et al.* 1992; Majiwa *et al.* 1994; Solano & Amsler-Delafosse, 1995; Reifenberg *et al.* 1997; Lefrançois *et al.* 1998), it is very costly to be used routinely on field samples. The

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detection of antigens by capture enzyme-linked immunosorbent assays (ELISA), using a monoclonal antibody directed against a 10 kDa polypeptide band from *T. vivax*, also have been shown to lack specificity and sensitivity (Desquesnes, 1996; Eisler *et al*. 1998). In addition, Rebeski *et al*. (1999) have reported that ELISA examinations are entirely unsuitable for the reliable detection of trypanosome antigens. Conversely, detection of anti-trypanosome circulating antibodies by ELISA, using a clarified antigenic fraction from *T. evansi*, has the advantage of being economical and applicable at a large scale (Reyna-Bello *et al*. 1998). More recently, Rebeski *et al*. (2000) also reported an appropriate and dependable ELISA test for anti-trypanosome antibody detection using microtitre plates pre-coated with heat and detergent denatured antigens from *T. congolense* and *T. vivax*. However, none of these serological assays define the antigens responsible for the immunological response. Thus, the improvement of a diagnosis test for the trypanosomiasis caused by salivarian trypanosomes represents a practical problem that requires to be worked out urgently. One approach that has been very useful in other diseases is the direct evaluation of purified antigens by serological methods, either singly or in groups.

Rodents can be experimentally infected with *T. evansi*, in order to obtain enough parasites to prepare *T. evansi* antigens for ELISA tests. On the contrary, the production of *T. vivax* antigens is a limiting factor in most laboratories (Desquesnes & Tresse, 1996). Nevertheless, *T. evansi* and *T. vivax* have shown a very high immunological cross-reactivity, and *T. evansi* antigens have been previously utilized to detect *T. vivax* (Reyna-Bello *et al*. 1998; Desquesnes & Tresse, 1996; Aray *et al*. 1998). In the present work, we monitored the major polypeptide bands from the Venezuelan TEVA1 isolate from *T. evansi* that showed cross-reaction with serum from equines and bovines infected with *T. evansi* and *T. vivax*, respectively. A 64 kDa antigen (p64) was isolated to homogeneity from *T. evansi* and was proven to be recognized by anti-*T. vivax* bovine antibodies. The evidence presented here indicates that p64 probably is the soluble form of a variable surface glycoprotein (VSG) from *T. evansi*.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following sources: middle range molecular weight protein markers, anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate, anti-mouse IgG (whole molecule) horseradish peroxidase conjugate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitroblue tetrazolium (NBT), Promega; anti-bovine IgG (whole molecule) horseradish peroxidase conjugate, anti-bovine IgG (whole molecule) alkaline phosphatase conjugate, anti-equine IgG (whole molecule) alkaline phosphatase conjugate, fluorescein-conjugated anti-bovine IgG (whole molecule), benzamidine, iodoacetamide, phenylmethyl sulfonyl fluoride (PMSF), fibrous DEAE-cellulose, diaminobenzidine (DAB), 2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide), peroxidase type VI-A (EC 1.11.1.7), Ponceau S red, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, Sigma; pre-stained high molecular weight protein markers, BRL; lipid A, Ribi Immunochemical Research; Q-Sepharose, S-Sepharose, concanavalin A, Pharmacia; fluorescein-conjugated anti-mouse IgG (whole molecule), Pierce; 96-well polypropylene plates (PolySorp), Nunc. All other reagents were analytical grade.

Source of antigens

T. evansi was obtained from a naturally infected horse from the Venezuelan savanna (Apure State). Initially, the parasites were expanded by inoculating 0·5 ml of horse-infected blood into 2 adult albino rats (Sprague Dawley). Further expansions of the parasites were carried out by inoculating 0·5 ml of rat infected blood containing approximately 10^8 parasites into other rats. This Venezuelan isolate of *T. evansi* was previously characterized by Desquesnes & Tresse (1996) using PCR, and was named TEVA1. When the number of parasites reached about 10^6–10^8 trypanosomes/ml, the blood was extracted from the rats by cardiac puncture using EDTA as anticoagulant. Parasites were purified by anion-exchange chromatography using a fibrous DEAE-cellulose column (Lanham & Godfrey, 1970). Parasites eluting from the column were collected by centrifugation at 1475 ***g***, for 20 min, at 4 °C, and washed 3 times with 20 mM phosphate buffer (pH 7·2) containing 1% glucose. The concentration of parasites was determined using a Neubauer chamber. The final cell pellet was kept frozen at −70 °C until further use.

T. evansi parasites (10^9) were extracted on ice, by sonication (2 cycles, 30 sec each, with a 30 sec resting period in between) using 2 ml of a 5 mM Tris–HCl buffer (pH 7·2) containing 1 mM benzamidine, 1 mM PMSF, 5 mM EDTA, and 1 mM iodoacetamide. The resulting homogenate was centrifuged at 15000 ***g*** for 30 min, at 4 °C, to obtain the supernatant and pellet fractions. The supernatant fraction was used as the source of parasite antigens, and was defined as the clarified antigenic fraction from *T. evansi*.

Experimentally infected animals

Two equines and 5 bovines were infected with 10^6 *T. evansi* or *T. vivax*, respectively. Two different

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T. evansi isolates were employed to contaminate the horses. The first horse was infected with the TEVA1 *T. evansi* isolate. The second horse was infected with a *T. evansi* isolate identified as 'TeAp-El Frio', which was obtained from a naturally infected capybara (*Hydrochoerus hydrochaeris*) from the Venezuelan savanna (Apure State, Venezuela). *T. vivax* was isolated from a naturally infected bovine from the Falcon State, Venezuela, and was generously donated by F. García. Inoculating bovine infected blood containing approximately 10^6 parasites into a goat allowed further expansions of *T. vivax*, which was subsequently purified by the isopycnic procedure on Percoll gradients reported by Grab & Bwayo (1982). Blood samples from experimentally infected animals were taken every day, for a 2-month period, in order to determine the parasitaemia by the micro-haematocrit method (Woo, 1970). Additionally, sera from animals naturally infected with trypanosomes were obtained and monitored for trypanosomiasis by indirect ELISA. Sera from healthy horses and cows also were obtained in order to use them as negative controls.

Purification of the p64 polypeptide band

T. evansi parasites ($2{\cdot}7 \times 10^{11}$) were resuspended in 40 ml of 5 mM Tris–HCl buffer (pH 7·2), 1 mM, benzamidine, 1 mM PMSF, 5 mM EDTA, 1 mM iodoacetamide, and sonicated as described above. The homogenate was centrifuged at 15000 ***g*** for 30 min at 4 °C. The resulting supernatant was loaded on a 50 ml Q-Sepharose column connected *in tandem* with a 10 ml S-Sepharose column, which previously had been equilibrated with 50 mM Tris–HCl (pH 7·2) containing the same protease inhibitors. The p64 polypeptide eluted in the flow-through fraction.

Preparation of polyclonal antibodies against the purified p64 polypeptide band

The purified p64 antigen was used to produce polyclonal antibodies in ascitic fluid from 7 female BalbC mice, according to the procedure described by Bubis, Millán & Martínez (1993). We pre-immunized the mice twice (at 7 and 9 weeks of age), under conditions that did not involve ascites fluid formation, using lipid A and muramyl dipeptide as adjuvants. Then, when the mice were 10 weeks old, we followed the standard protocol described by Tung *et al*. (1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

Protein gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS) on 1·5 mm thick slab gels containing 12% polyacrylamide (Laemmli, 1970). All gels were run at constant current (45 mA). Coomassie blue R-250 was used for protein staining.

Western blot analyses

Following SDS–PAGE, the proteins were electrotransferred from the gels to nitrocellulose sheets (0·45 μm pore size) using the procedure described by Towbin, Staehelin & Gordon (1979). For immunodetection, the nitrocellulose filters were incubated with bovine or equine serum (dilutions 1:100–1:1000), or with specific mouse polyclonal antibodies to p64 (dilution 1:10000). The sheets were then 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. Finally, the polypeptide 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. A lane containing a mixture of molecular weight protein markers was included in the blot to determine the apparent size of the polypeptide bands. This lane was cut and stained with Ponceau S red. In some cases, pre-stained protein markers were used.

Detection of concanavalin A-binding glycoproteins

Following SDS–PAGE, the purified antigen was electrotransferred from the gels to nitrocellulose membranes as described above. For the detection of glycoproteins, the protocol reported by Wood & Sarinana (1975) was utilized. Briefly, the nitrocellulose membrane was initially placed in a PBS solution (20 mM phosphate buffer (pH 7·2), 150 mM NaCl) containing 0·1% Tween 20 and 1% gelatin, for 1 h. It was then incubated for 45 min with concanavalin A (0·5 mg/ml) in PBST buffer (PBS containing 0·1% Tween 20). After 3 × 10 min washes with PBST, the nitrocellulose sheet was incubated for 45 min with horseradish peroxidase (0·1 mg/ml in PBST), and the colour reaction was developed with DAB and hydrogen peroxide and stopped with an excess of distilled water. Parallel experiments in which the nitrocellulose membranes were treated with concanavalin A, previously incubated with 0·2 or 0·5 M methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, or a mixture of both carbohydrates, were included as controls.

Detection of a glucosyl-phosphatidylinositol (GPI) moiety on p64

The GPI attachment on p64 was indirectly detected by Western blot using a polyclonal antibody

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produced in rabbits against the cross-reacting determinant (CRD) of the hydrophilic gp63 from *Leishmania mexicana* (Bahr *et al*. 1993). CRD appears after lysis of the GPI anchor by a GPI-specific phospholipase C. This polyclonal antibody was a generous gift of D. Sterverding who used it for the detection of CRD on *T. brucei* VSG (Sterverding & Kremp, 1998). This polyvalent antiserum was used at a 1:700 dilution in TBST buffer (50 mM Tris–HCl, pH 8·0, 150 mM NaCl, 0·1 % Tween 20). Then, the blot was incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibodies against rabbit IgG, and the protein band was visualized by the addition of DAB and hydrogen peroxide.

Mild acid treatment

Samples containing 1 μg of p64 each were treated with 1 M HCl, for 2 h, at room temperature, as described by Schneider *et al*. (1990). After neutralization with NaOH, the samples were prepared for SDS–PAGE, separated by electrophoresis, and electroblotted onto nitrocellulose paper. The membranes were revealed with sera from animals naturally or experimentally infected with trypanosomes, at 2 different dilutions (1:500 and 1:1000). The assay included sera from 2 equines and 2 bovines experimentally infected with *T. evansi* and *T. vivax*, respectively, from 6 cows and 1 buffalo naturally infected with *T. vivax*, and from a donkey naturally infected with *T. evansi*. As a control, parallel experiments were developed with anti-CRD of gp63 from *L. mexicana* rabbit antiserum.

Indirect immunofluorescence

The blood of *T. evansi*-infected Sprague Dawley rats (10^6 parasites/ml) 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 1 volume of 2 % BSA in PBS*, and aliquots of 10 μl were smeared on microscope slides. Immunofluorescence was carried out following the procedure described by Cons & Kaplan (1950). Microscope slides pre-coated with blood containing *T. evansi* parasites, were incubated with different dilutions of sera obtained from the experimentally infected bovines. Mouse polyclonal antibodies raised against the purified p64 from *T. evansi* (dilution 1:160) also were used to determine the subcellular localization of this polypeptide by immunofluorescence. Then, a fluorescein-conjugated secondary antibody against bovine or mouse IgG (dilution 1:50) was employed. The parasites were viewed using a fluorescence microscope directly connected with a photographic camera.

Indirect ELISA

Indirect ELISA was carried out according to the method described by Aray *et al*. (1998). Concisely, ELISA plates were sensitized with the clarified antigenic fraction from *T. evansi* (1 μg of protein/well), diluted in carbonate–bicarbonate buffer (pH 9·6), overnight at 4 °C in a humid chamber. Then, blocking buffer (PBS containing 0·1 % Tween 20 and 2 % gelatin) was applied in excess to each well for 1 h at 37 °C. Bovine sera from experimentally or naturally infected animals were diluted 1:100 in blocking buffer, and a 100 μl aliquot of the diluted serum was added per well. After an extensive wash, a horseradish peroxidase-conjugated secondary antibody against bovine IgG (dilution 1:2000, in blocking buffer) was supplemented using 100 μl/well. The colour 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.

Protein determination

Protein concentration was determined as reported by Bradford (1976), using bovine serum albumin as protein standard.

RESULTS

T. evansi *antigens identified by sera from infected animals*

Sera from horses experimentally infected with *T. evansi* were used to identify *T. evansi* antigenic bands by Western blots. As described above, 2 different *T. evansi* isolates were used to infect the horses. The first horse (Fig. 1A) was infected with the TEVA1 isolate, while the second horse (Fig. 1B) was infected with *T. evansi* isolated from a capybara. Interestingly, both animals developed the clinical trypanosomiasis symptoms (data not shown). Fig. 1 shows that increasing numbers of trypanosome components were recognized, and a more evident and intense staining of individual polypeptide bands was produced as the infection advanced in time. As seen in the figure, the equine antibodies in both cases (A and B) recognized approximately the same protein pattern, and the apparent molecular masses of the *T. evansi* components ranged from approximately 14 to 109 kDa. Seventeen major polypeptide species (109, 104, 86, 82, 79, 73, 68–60, 58, 53, 46, 44, 40, 32, 31, 17, 15, and 14 kDa) were identified by the 2 equine sera (Fig. 1A and B). Western blot analyses were also performed to identify the antigenic components from *T. evansi*, which were recognized by sera from 2 bovines infected with *T. vivax*. Fig. 1 illustrates that an identical polypeptide pattern to that shown with equine sera (A and B) was obtained when

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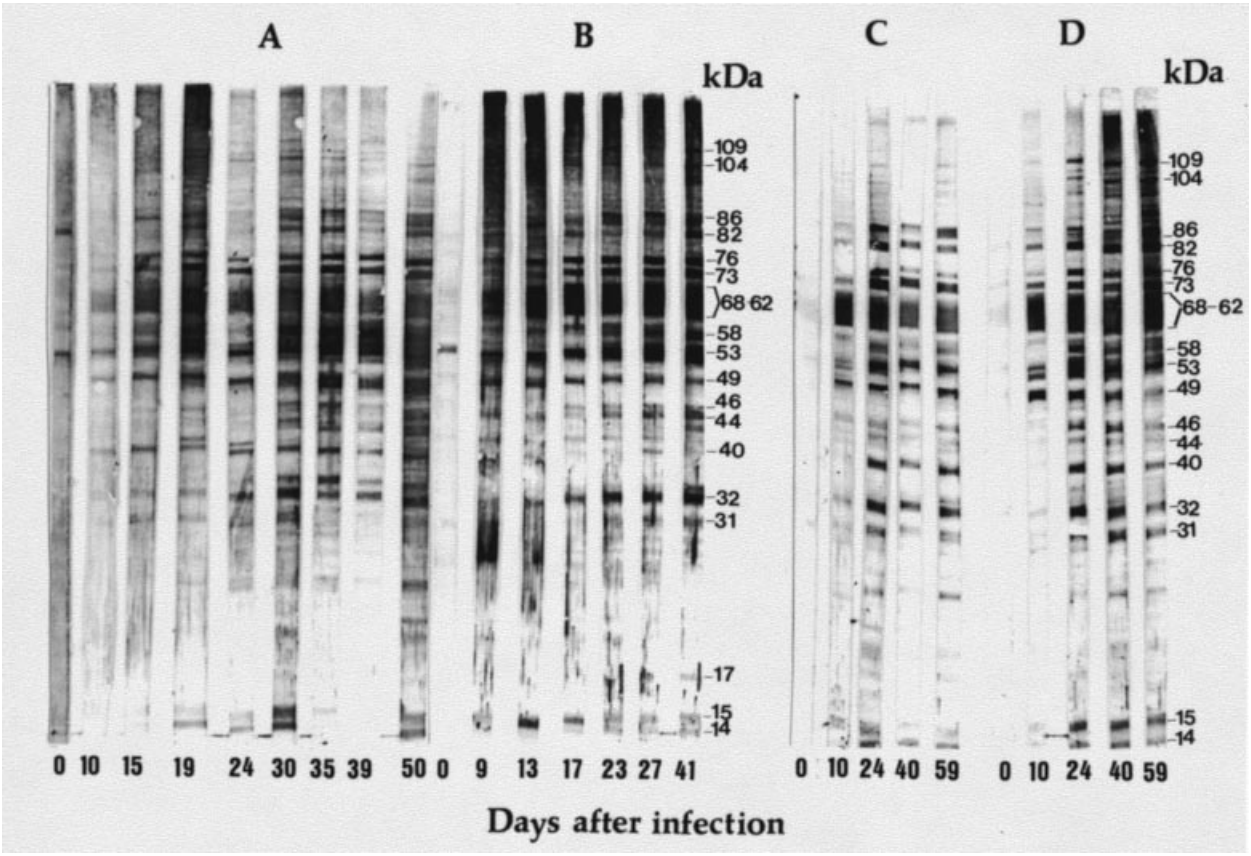

Fig. 1. Identification of antigenic bands from *Trypanosoma evansi* by Western blots. Aliquots of the clarified antigenic fraction from *T. evansi*, containing 300 μg of total protein, were separated by SDS–PAGE and electrotransferred to nitrocellulose sheets. Panels A and B show the polypeptide bands recognized by serum from horses infected with *T. evansi*. The first horse (A) was infected with the TEVA1 isolate, while the second horse (B) was infected with *T. evansi* (TeAp-EI Frio) isolated from a capybara. (C and D) Immunoblots developed using sera from 2 different bovines infected with a Venezuelan *T. vivax* isolate. Shown are the apparent molecular masses of the major antigens. The days after infection are also indicated.

bovine serum was used to develop the blots (C and D).

Indirect immunofluorescence microscopy assay to monitor the reaction of T. evansi *with serum from bovines infected with* T. vivax

Indirect immunofluorescence was used in order to localize on *T. evansi* the antigens responsible for the cross-reactivity with bovine anti-*T. vivax* antibodies. As shown in Fig. 2A, sera from bovines infected with *T. vivax* were capable of effectively recognizing blood-stream *T. evansi* parasites. A strong immunological response was detected on the cell surface and the nucleus of *T. evansi*, indicating that the antigens, which are involved in the cross-reaction between *T. evansi* and *T. vivax* must be predominantly located on the cellular membrane and proximal to the nucleus of the parasites.

Purification and characterization of p64 from T. evansi

The clarified antigenic fraction from *T. evansi* was chromatographed on a Q-Sepharose column connected in series with a S-Sepharose column. Both resins were washed *in tandem* with 50 mM Tris–HCl (pH 7·2) containing protease inhibitors. A prominent non-absorbing protein peak was obtained (Fig. 3A). SDS–PAGE analysis of the fractions comprising the flow-through material from both columns showed the presence of a 64 kDa polypeptide (Fig. 3B). After pooling fractions 2–9 from the chromatograph (Fig. 3A), p64 was 99% pure by the criteria of SDS–PAGE (data not included). This procedure yielded 0·62 mg of p64 starting from $2{\cdot}7 \times 10^{11}$ parasites. The p64 polypeptide represents approximately 7% of the total parasite protein loaded onto the columns, indicating that it is a major component of *T. evansi*.

The 64 kDa polypeptide is present in both the soluble and the particulate fraction of T. evansi *and has antigenic determinants recognized by equine anti-*T. evansi *and bovine anti-*T. vivax *antibodies*

Fig. 4 shows the Coomassie blue staining (A) and the immunostaining using sera from horses infected with *T. evansi* (B) and cows infected with *T. vivax* (C), of the total *T. evansi* homogenate (lane H), the parasite soluble fraction (lane S), the parasite

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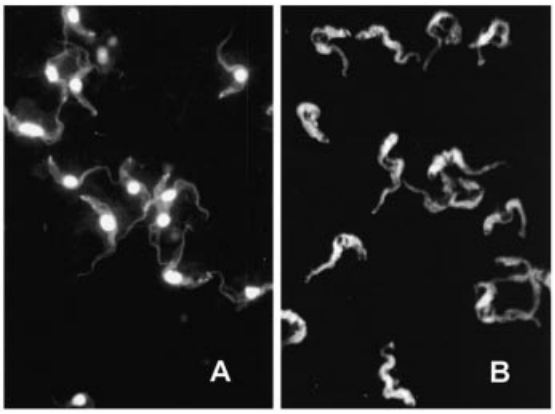

Fig. 2. (A) Cross-reactivity between *Trypanosoma evansi* and *T. vivax* determined by immnuofluorescence. Slides pre-coated with blood-stream *T. evansi* parasites were incubated with sera obtained from a cow infected with *T. vivax*. A fluorescein-conjugated secondary antibody against bovine IgG was employed as described in the Materials and Methods section. The parasites were viewed using a fluorescence microscope directly connected with a photographic camera.
(B) Immunofluorescence microscopy analysis of the cellular localization of *T. evansi* p64. Indirect immunofluorescence labeling reveals the presence of p64 on the whole *T. evansi* cell except for the nucleus. Magnification = 800 ×.

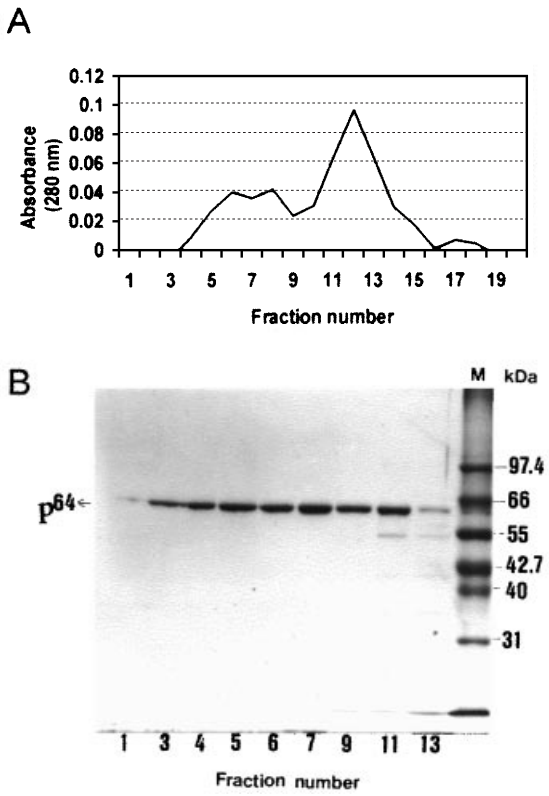

Fig. 3. Purification of the 64 kDa antigen from *Trypanosoma evansi*. The clarified antigenic fraction from *T. evansi* was chromatographed on a Q-Sepharose column connected *in tandem* with a S-Sepharose column. The elution profile of the non-absorbing material, determined by absorbance at 280 nm, is represented in (A). The flow-through fractions separated by SDS–PAGE are shown in (B). The gel was stained with Coomassie blue. M = protein markers. The arrow shows the migration of the p64 antigen.

particulate fraction (lane P), and the isolated p64 polypeptide. As illustrated in Fig. 4, p64 corresponds to a major polypeptide species of *T. evansi*, which is present in both the cytosol and the particulate parasite fraction. Interestingly, purified p64 was recognized by both equine anti-*T. evansi* antibodies and bovine anti-*T. vivax* antibodies.

To provide a tool for the identification of the 64 kDa antigen, a polyclonal antibody was raised against the purified protein in mice ascitic fluid. Indirect ELISA evaluation yielded a titre of 1:30000 for the antibodies prepared in the ascites (data not shown). This antibody specifically recognized the 64 kDa band by immunoblots on nitrocellulose membranes (Fig. 4D). Following the homogenization procedure employed here, the anti-p64 polyclonal antibodies were employed to specifically examine the presence of p64 in each resulting subcellular fraction. Although p64 was present in both the cytosol and the particulate parasite fraction, the figure shows that p64 was mostly located in the soluble fraction, while some p64 was maintained in the particulate portion of the parasite.

Localization of the 64 kDa antigen on T. evansi *by immunofluorescence microscopy*

The polyclonal antibodies directed against p64 were also utilized to locate the antigenic protein on the parasite cell, by immunofluorescence microscopy. Anti-p64 polyclonal antibodies heavily labelled the whole parasite including the flagella (Fig. 2B). However, no fluorescence was obtained on the cell nucleus. Furthermore, no labelling was observed when fluorescein-conjugated anti-mouse IgG was employed alone (i.e. without pre-treating the sections with the specific antibodies against p64), or when another ascitic fluid produced against the γ subunit of bovine transducin, a non-related protein, was used (data not shown).

The 64 kDa polypeptide contains a GPI membrane attachment

As shown in Fig. 4E, purified p64 was strongly detected by rabbit antiserum generated against CRD of the hydrophilic gp63 from *Leishmania mexicana*. The p64 polypeptide was also recognized in *T. evansi* whole-cell homogenates (H), and soluble (S) and particulate (P) fractions, by the anti-CRD antibodies (Fig. 4E). Accordingly, native p64 must contain a GPI moiety similar to that observed in gp63 from *L. mexicana* and in VSG from *T. brucei*. Since the anti-CRD antibodies were raised against the soluble form of the *L. mexicana* gp63, they should not react with the membrane-bound form of

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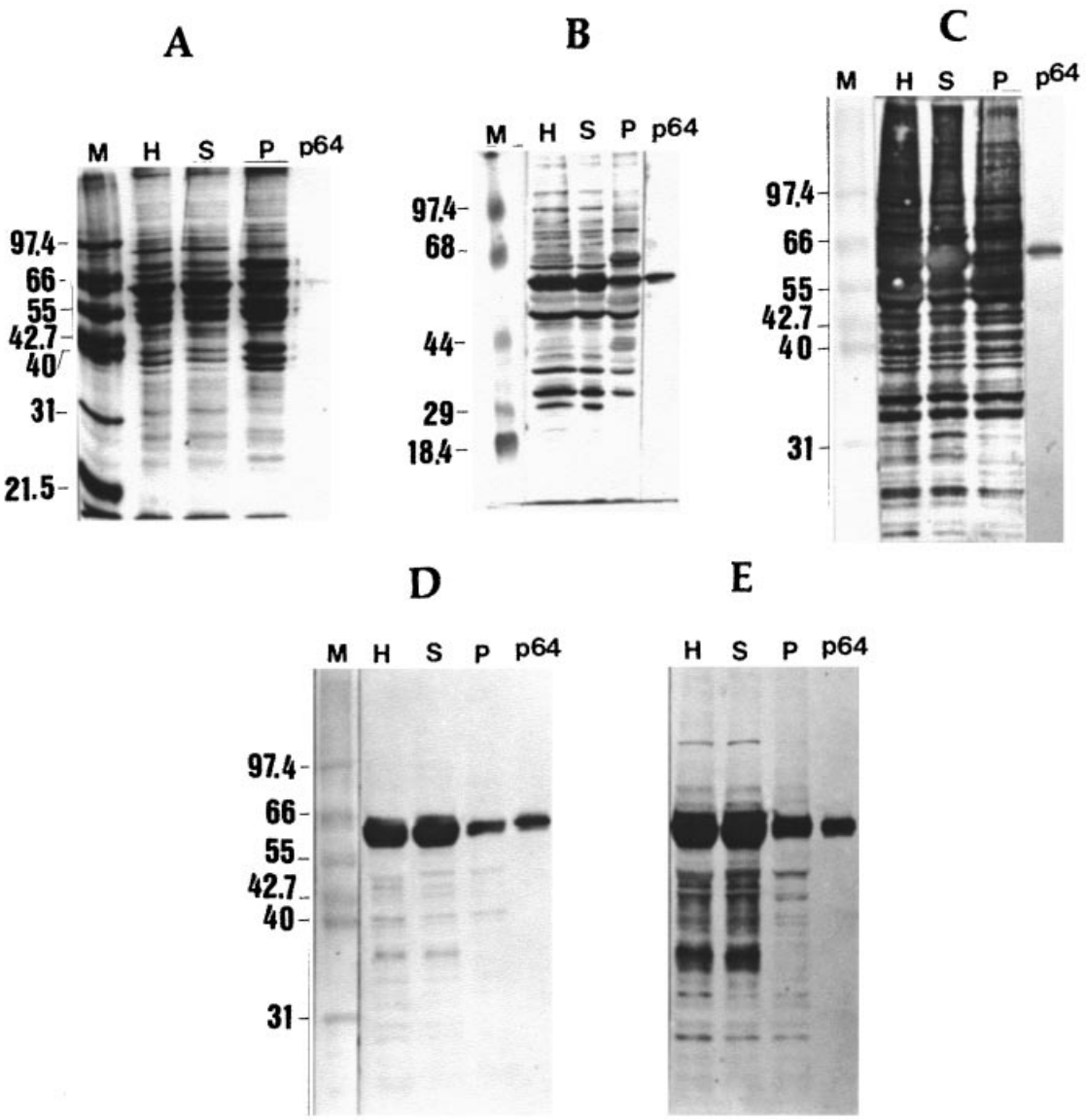

Fig. 4. Localization, cross-reactivity with bovine anti-*Trypanosoma vivax* antibodies, and recognition by anti-CRD antibodies of p64 from *T. evansi*. *T. evansi* parasites were extracted by sonication. The resulting homogenate (H) was centrifuged and supernatant (S) and pellet (P) fractions were obtained. (A) Coomassie blue staining; (B) immunoblot developed with equine serum obtained 30 days after infection with *T. evansi* (sample used in Fig. 1A); (C) immunoblot developed with bovine serum obtained 24 days after infection with *T. vivax* (sample used in Fig. 1C); (D) immunoblot developed using anti-p64 polyclonal antibodies; (E) immunoblot developed using anti-CRD antibody. M = protein markers, p64 = purified 64 kDa antigen from *T. evansi*.

p64. However, as seen in Fig. 4E (P), the pellet-associated p64 was reactive with anti-CRD antibodies. This result suggests that some cleavage of the p64 remaining in the particulate fraction has taken place prior to running the gel, probably caused by an endogenous GPI-phospholipase C-like activity.

P64 is a glycoprotein

As shown in Fig. 5, p64 specifically interacts with concanavalin A. Methyl-α-D-mannopyranoside (MM) and methyl-α-D-glucopyranoside (MG) at concentrations of 0·2 and 0·5 M, both individually and combined, competed with the recognition of the lectin. However, MM inhibited concanavalin A binding to p64 more efficiently than MG. These results demonstrated that p64 is a glycoprotein enriched in α-D-mannose and/or α-D-glucose.

Anti-p64 antibodies recognized a 53 kDa polypeptide band in T. vivax *homogenates*

Since sera from bovines infected with *T. vivax* recognized p64 from *T. evansi* by Western blots, we also examined whether the anti-p64 polyclonal antibodies, raised in mice, were capable of recognizing polypeptide bands in *T. vivax* whole homogenates. As shown in Fig. 6, antibodies against p64 recognized a 53 kDa polypeptide in extracts of a Venezuelan isolate of *T. vivax*.

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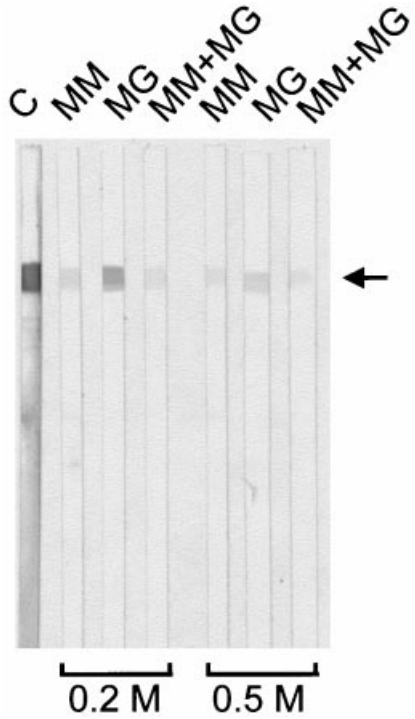

Fig. 5. The p64 polypeptide band is a glycoprotein. Purified p64 (50 μg) was separated on a preparative slab mini-gel by SDS–PAGE and electrotransferred to a nitrocellulose filter, which was cut in 4 mm-strips each. The strips were treated with concanavalin A (C), or concanavalin A pre-incubated with methyl-α-D-mannopyranoside (MM), methyl-α-D-glucopyranoside (MG), or the mixture of both carbohydrates (MM+MG), at concentrations of 0·2 and 0·5 M. The arrow indicates the migration of p64.

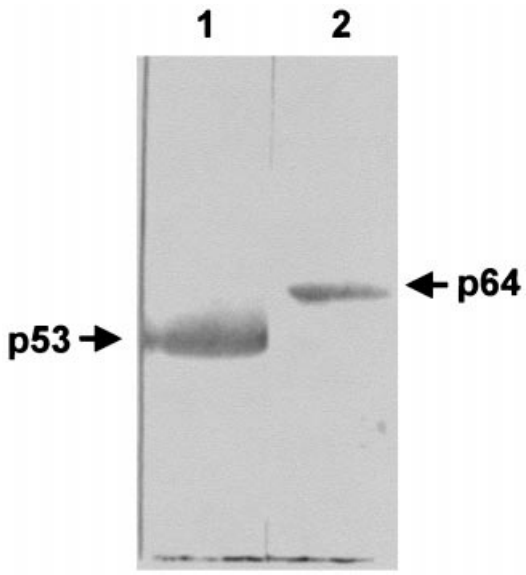

Fig. 6. Cross-reactivity of anti-p64 polyclonal antibodies with a 53 kDa polypeptide band in *Trypanosoma vivax* whole cell extract. Purified p64 from *T. evansi* (1) and an aliquot of a *T. vivax* homogenate (2) were subjected to SDS–PAGE and analysed by Western blot using anti-p64 polyclonal antibodies. The arrows indicate the migration of p64 and the 53-kDa polypeptide from *T. vivax*, which was immunorecognized by anti-p64 antibodies.

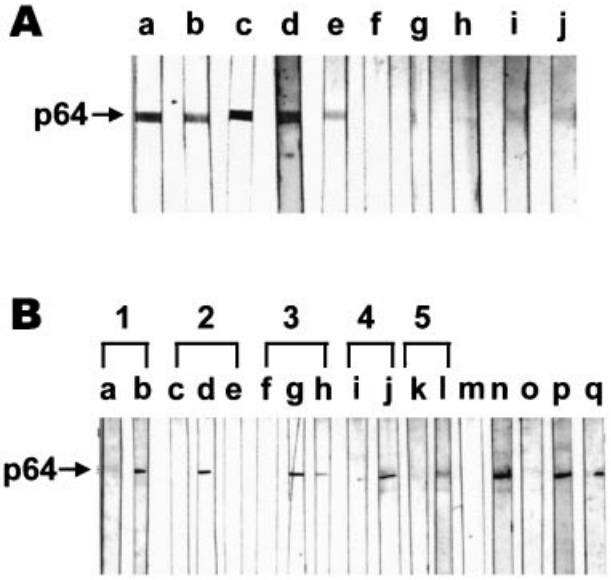

Fig. 7. Reactivity of the p64 polypeptide with sera from horses and cows infected with *Trypanosoma evansi* and *T. vivax*, respectively. The 64 kDa polypeptide was separated by SDS–PAGE and electroblotted onto nitrocellulose. The blots were cut into 4 mm strips. (A) Strips were developed using sera from the following sources: horses experimentally infected with *T. evansi* (lanes a and b, which correspond to the samples used in Fig. 1A and B, respectively); naturally infected equines diagnosed as positive for trypanosomiasis by indirect ELISA (lanes c–e); and healthy racehorses living in a trypanosomiasis-free area at La Rinconada Racetrack, and determined to be negative to trypanosomiasis by indirect ELISA (lanes f–j). (B) Strips were developed using sera from 5 bovines experimentally infected with *T. vivax* (1–5; 2 and 3 being the same animals used in Fig. 1C and D). Lanes a, c, f, i and k; strips developed with bovine sera before trypanosome infection. Lanes b, d, g, j and l; strips developed with sera of the same animals, obtained 20–30 days after infection with *T. vivax*. Lanes e and h; strips developed using sera from cows 2 and 3 after 1 year of treatment with curative doses of trypamidium. Also shown are healthy bovines determined to be negative to trypanosomiasis by indirect ELISA (lanes m and o), and naturally infected cows diagnosed as positive for trypanosomiasis by indirect ELISA (lanes n, p and q).

The 64 kDa polypeptide is recognized by equine and bovine sera of naturally infected animals

Western blots were utilized in order to evaluate the recognition of p64 by sera from naturally infected equines and bovines. Purified p64 was separated by SDS–PAGE and electrotransferred to nitrocellulose filters. Then, sera from horses and cows diagnosed as positive for trypanosomiasis by indirect ELISA (data not shown), as well as sera from healthy animals, were used to determine their immunological response against p64. As shown in Fig. 7, p64 was immunorecognized by sera obtained from equines (A, lanes c–e) and bovines (B, lanes n, p and q), which had been naturally infected with *T. evansi* and

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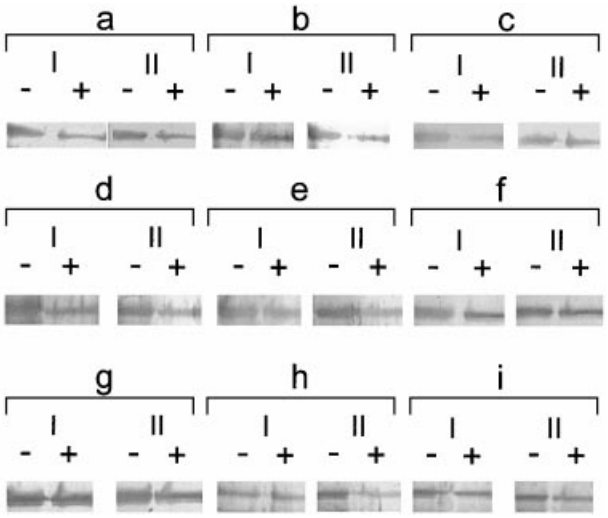

Fig. 8. Effect of mild acid treatment on the cross-reaction of p64 with sera from infected animals. The 64 kDa polypeptide (1 μg) was incubated without (−) or with 1 M HCl (+), for 2 h at room temperature. Sera from 6 bovines (a–f), a buffalo (g) and a donkey (h), all naturally infected with trypanosomes, as well as serum from the experimentally infected bovine shown at Fig. 1C (i), were used to monitor the CRD-dependent cross-reactivity of p64, at dilutions of 1:500 (I) and 1:1000 (II).

T. vivax, respectively, but not by sera from uninfected animals (A, lanes f–j; B, lanes a, c, f, i, k, m and o). For comparison, sera from experimentally infected animals were also included in the experiment (Fig. 7A, lanes a and b; Fig. 7B, lanes b, d, g, j and l). Interestingly, sera from experimentally infected cows, which have been treated for 1 year with curative doses of trypamidium, strongly decrease the recognition of p64 (Fig. 7B, lanes e and h). Moreover, Fig. 8 illustrates the recognition of p64 by sera obtained from 6 bovines different from those used in Fig. 7 (a–f), a buffalo (g), and a donkey (h), which have been naturally infected with trypanosomes. A high immunological response of the various animals' sera against p64 was observed even at dilutions of 1:1000 (Fig. 8, II). All these results suggest a potential use of p64 as a diagnostic reagent for animal trypanosomiasis.

The cross-reactivity of p64 is only partial via the inositol-1,2-cyclic phosphate moiety of CRD

Sera from 7 bovines, a buffalo, and a donkey, which had been infected with trypanosomes, were used to monitor the CRD-dependent cross-reactivity of p64. Incubation of the 64 kDa polypeptide with 1 M HCl for 2 h, slightly decreased the recognition of the antigen by each serum, at dilutions of 1:500 and 1:1000 (Fig. 8). Since mild acid treatment has been reported to produce the decyclization of the inositol-1,2-cyclic phosphate moiety of CRD from proteins *in vitro* (Zamze *et al*. 1988; Schneider *et al*. 1990), these results indicated that the inositol structure is not completely responsible for the antigenicity and cross-reactivity of p64. Therefore, another cross-reacting epitope must exist in this polypeptide. Similarly, the recognition of p64 by anti-CRD rabbit antiserum was also partially acid sensitive (data not shown). Incubation with acid (up to 2 h) did not hydrolyse the p64 polypeptide as demonstrated by SDS–PAGE followed by Coomassie blue staining (data not included).

DISCUSSION

T. evansi and *T. vivax* have shown a very high immunological cross-reactivity, which has been previously reported using capillary agglutination (Toro *et al*. 1980), and indirect ELISA (Aray *et al*. 1998; Reyna-Bello *et al*. 1998). Our findings using Western blot and immunofluorescence microscopy reiterate the immunological cross-reaction between these two trypanosomes. In the present work, we have identified some antigens responsible for this cross-reactivity between *T. evansi* and *T. vivax*, by immunoblotting. A series of polypeptide species present in the clarified soluble antigenic fraction of *T. evansi*, ranging from 14 to 109 kDa, were common antigens for both anti-*T. evansi* equine antibodies and anti-*T. vivax* bovine antibodies. Previously, Uche, Jones & Boid (1993) have reported the polypeptide pattern recognized by specific antibodies prepared in rabbits infected with *T. evansi* and observed antigens of 85, 67, 60·5, 43, and 32·4 kDa until 28 days after infection. Although we found more antigenic polypeptides, our results were consistent with those of Uche *et al*. (1993).

Among the *T. evansi* antigens, a 64 kDa band was very representative, corresponding to about 7% of the total protein from the clarified cytosolic fraction from the parasite. Here, we report the purification to homogeneity of this 64 kDa polypeptide from *T. evansi*, which is the first antigen isolated and partially characterized from this parasite to date. The p64 antigen was also recognized by sera obtained from bovines infected with *T. vivax*, being then partially responsible for the cross-reaction between *T. evansi* and *T. vivax*. Despite the presence of p64 in the parasite soluble fraction, immunofluorescence experiments using mouse polyclonal anti-p64 antibodies followed by fluorescein-conjugated anti-mouse IgG, seem to localize the native protein on the whole cell but not on the nucleus, suggesting that this polypeptide could be primarily localized on the parasite surface. Apparently the sonication procedure, normally used to homogenize the parasites, somehow released p64 into the cytosol, converting the putative membrane-bound form of p64 into a soluble form. However, some p64 remained in the

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particulate fraction. Formerly, Uche, Ross & Jones (1992) have used ^{125}I-labelling to characterize the surface components of *T. evansi* and found 2 components of apparent molecular mass of 67 and 60·5 kDa, which were labelled in some of the parasite stocks. The p64 polypeptide probably corresponds to one of the components labelled and reported by Uche *et al*. (1992). Immunofluorescence microscopy was also used here to examine the localization of the cross-reacting antigens on *T. evansi*. The immunofluorescence results obtained using anti-*T. vivax* bovine antibodies indicated that the major cross-reacting antigens of *T. evansi* are localized either on the cell surface or proximal to the nucleus of the parasite. Then, the p64 antigen could be partially responsible for the recognition observed on the surface of the parasite. Consistent with all these observations, p64 is a glycoprotein as was evidenced by concanavalin A binding.

A GPI membrane anchor on p64 was indirectly detected by immunoblots. This moiety probably functions linking the p64 antigen onto the surface of the parasite. The outer layer of salivarian trypanosomes consists of a coat of identical glycoprotein molecules the chemical composition of which changes throughout the course of infection in the vertebrate host (Vickerman, 1969). As in other parasites, the host response to the trypanosomes surface antigen plays an important role in controlling the infection. Protective antibodies are surface specific but the process of antigen variation compromises their effectiveness (Uche *et al*. 1992). The variability observed on the variable surface glycoproteins or VSGs is a major strategy of the salivarian trypanosomes to evade the immune system of the host. These proteins represent the major protein component in *T. brucei* and other salivarian trypanosomes. In these trypanosomes, VSGs are secondarily modified by the addition of carbohydrate side-chains (Holder, 1985) and a complex GPI moiety containing dimyristylglycerol and carbohydrate. GPI is linked via ethanolamine to the carboxy-terminal amino acid residue (Ferguson *et al*. 1988). Both the complex GPI and a glycolipid tail act as membrane anchors of the membrane-bound form of VSG (Turner *et al*. 1985). The direct solubilization of trypanosomes in detergent leads to the recovery of this form of VSG. However, for *T. brucei*, hypotonic lysis causes the release of an endogenous phospholipase C-like activity, which cleaves the glycolipid anchor and allows the release of the remainder of the molecule in soluble form. Likewise, *T. evansi* probably contains an endogenous phospholipase C-like activity, which would be responsible of releasing the membrane-bound form of p64 into the cytosol. Moreover, p64 may correspond to the soluble form of the putative VSG from *T. evansi*, on the basis of its relative abundance, its probable location on the parasite surface, its release into the parasite soluble fraction following extraction, the presence of carbohydrate and the GPI moiety on its structure, and its apparent molecular weight by SDS–PAGE, which is in the range of reported values for VSGs from *T. brucei*, an evolutionarily related parasite (Turner *et al*. 1985). If this is the case, the yield obtained here for p64 was low compared to typical VSG yields from *T. brucei*, which have ranged from 30 to 60 mg per 10^{11} cells (Cross, 1984). Since the amount of VSG on *T. evansi* has not been estimated yet, the simplest explanation may perhaps be that *T. evansi* contains less VSG molecules than *T. brucei*. Additionally, the procedure used to prepare the *T. evansi*-soluble fraction was performed on ice which, in *T. brucei*, limits the action of the endogenous phospholipase C-like activity on the membrane-bound form of VSG. This may also explain to some extent the low p64 yield. An improvement on the release of the soluble form of *T. evansi* VSG, and concomitantly of p64, may possibly be induced by stimulating the phospholipase C-like activity of the parasite, as has been described for *T. brucei* (Cross, 1984; Steverding & Kremp, 1998).

Equine anti-*T. evansi* and bovine anti-*T. vivax* antibodies immunorecognized p64 of *T. evansi*. If p64 is the soluble form of a VSG from *T. evansi*, the variable region on the VSG molecule cannot be responsible of the observed recognition. In *T. brucei*, an epitope located on the anchoring moiety, which essentially has been defined by antibody reactivity, is present in all VSGs examined (Barbet & McGuire, 1978). As most VSGs are serologically unique, this cross-reacting determinant or CRD is generally the only part of the molecule to show such cross-reactivity (Gardiner *et al*. 1996). Bülow, Nonnengässer & Overath (1989) have demonstrated a high VSG turnover during the culture of bloodstream forms of *T. brucei in vitro*. Interestingly, VSGs naturally released into the medium can be immunoprecipitated with antibodies raised against CRD, and have the characteristics of the soluble form of VSG, as judged by its electrophoretic mobility. Then, it is likely that p64, which probably corresponds to the VSG from *T. evansi*, would be released during parasite infection. This could result in the production of antibodies against CRD by the animals. The classical clarified antigenic fraction used as a diagnostic test is obtained by mechanical disruption, and probably also contains soluble *T. evansi* VSG (p64). Consequently, the anti-CRD antibodies present in the sera from the infected animals could be capable of recognizing the soluble VSG. In an attempt to define the cross-reacting epitopes on p64, sera from trypanosome-infected animals were used here to monitor the CRD-dependent cross-reactivity of this antigen. A significant immunorecognition was retained when p64 was incubated with acid. Likewise, acid treatment decreased but did not eliminate the recognition of p64

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by rabbit antiserum generated against CRD of the hydrophilic gp63 from *L. mexicana*. Mild acid reaction has been shown to produce the decyclization of the inositol-1,2-cyclic phosphate moiety of CRD from proteins *in vitro* (Zamze *et al*. 1988; Schneider *et al*. 1990). Hence, the inositol structure seems to be only partially responsible for the antigenicity and cross-reactivity of p64. GPI moieties of other eukaryotic proteins have proven to contain α-galactose side-chains, which are resistant to mild acid treatment and are responsible for a significant recognition by anti-CRD antibodies (Zamze *et al*. 1988). Accordingly, the GPI anchor of p64 may contain galactose branches, which may perhaps be recognized by sera from trypanosome-infected animals. However, since gp63 from *L. mexicana* contains no galactose residues, other cross-reacting epitopes must be responsible for the remaining recognition of acid-treated p64 by the anti-CRD rabbit antiserum. Similarly, these additional cross-reacting epitopes of p64 may also cause some of its cross-reactivity with *T. vivax*. Interestingly, VSG has also been described for a *T. vivax* Uganda isolate (Gardiner *et al*. 1996). In this case, VSG is a small variable glycoprotein with an apparent molecular mass of 40 kDa. The reported *T. vivax* VSG bears little carbohydrate and also contains a GPI anchor (Gardiner *et al*. 1996). As shown here, anti-p64 polyclonal antibodies recognized a 53 kDa polypeptide band on whole extracts of a Venezuelan *T. vivax* isolate by Western blot analysis. The molecular weight discrepancy is typical of the proteins belonging to the VSG family, and suggests that we are dealing with a different *T. vivax* isolate than the African one. These phenomena have been reported for other trypanosomes. For example, the VSGs of *T. brucei* exhibit molecular masses of 55–67 kDa (Turner *et al*. 1985) and those of *T. congolense* fall in the range of 49–58 kDa (Rausch *et al*. 1994).

The work reported here represents only the beginning of the task of defining and purifying the antigenic proteins from *T. evansi*, which are also responsible for the immunological cross-reaction with *T. vivax*. The p64 polypeptide is the first purified *T. evansi* antigen available. It is easy to purify, and is recognized by sera from horses and cows infected with *T. evansi* and *T. vivax*, respectively. Consequently, p64 is a good candidate to be assessed for diagnostic purposes. The specificity and sensitivity of p64 are parameters being evaluated at the present time, in order to establish the possibility of utilizing p64 as a tool for the serological diagnosis of animal trypanosomiasis.

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REFERENCES

APPLEWHAITE, L. M. (1990). Small ruminant trypanosomiasis in Guyana – a preliminary report. *British Veterinary Journal* **146**, 93–94.

ARAY, C., UZCANGA, G., SOTO, H. & MENDOZA, M. (1998). Ensayo immunoenzimático para el diagnóstico de la tripanosomiasis bovina causada por *Trypanosoma* sp. Seroprevalencia en el municipio Monagas del Estado Guárico-Venezuela. *Revista Científica, Universidad del Zulia* **8**, 114–116.

BAHR, V., STIERHOF, Y.-D., ILG, T., DEMAR, M., QUINTEN, M. & OVERATH, P. (1993). Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of *Leishmania mexicana*. *Molecular and Biochemical Parasitology* **58**, 107–122.

BARBET, A. F. & MCGUIRE, T. C. (1978). Crossreacting determinants in variant-specific surface antigens of African trypanosomes. *Proceedings of the National Academy of Sciences, USA* **75**, 1989–1993.

BAUER, B., AMSLER-DELAFOSSE, S., CLAUSEN, P. H., KABORE, I. & PETRICH-BAUER, J. (1995). Successful application of deltamethrin poured on to cattle in a campaign against tsetse flies (*Glossina* spp.) in the pastoral zone of Samorogouan, Burkina Faso. *Tropical Medicine and Parasitology* **46**, 183–189.

BOYD, R. & MLECHE, W. C. H. (1985). Isoenzyme analysis of stocks of trypanosomes isolated from cattle in Indonesia. *Research in Veterinary Science* **39**, 388–389.

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

BUBIS, J., MILLÁN, E. J. & MARTÍNEZ, R. (1993). Identification of guanine nucleotide binding proteins from *Trypanosoma cruzi*. *Biological Research* **26**, 177–188.

BÜLOW, R., NONNENGÄSSER, C. & OVERATH, P. (1989). Release of the variant surface glycoprotein during differentiation of bloodstream to procyclic forms of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **32**, 85–92.

CONS, A. H. & KAPLAN, M. H. (1950). Localization of antigen in tissue cells. II. Improvements in a method for detection of antigen by means of fluorescent antibody. *Journal of Experimental Medicine* **91**, 1–13.

CROSS, G. A. M. (1984). Release and purification of *Trypanosoma brucei* variant surface glycoprotein. *Journal of Cellular Biochemistry* **24**, 112–122.

DESQUESNES, M. (1996). Evaluation of three antigen detection tests (Monoclonal trapping ELISA) for African Trypanosomes, with an isolate of

Trypanosoma vivax from French Guyana. *Annals of the New York Academy of Sciences* **791**, 172–184.

DESQUESNES, M. & TRESSE, L. (1996). Evaluation of sensitivity of PCR for detecting DNA of *Trypanosoma vivax* with several methods of blood sample preparations. *Revue Élevage et de Medecine Veterinaire des Pays Tropicaux* **49**, 322–327.

EISLER, M. C., LESSARD, P., MASAKE, R. A., MOLOO, S. K. & PEREGRINE, A. S. (1998). Sensitivity and specificity of antigen-capture ELISAs for diagnosis of *Trypanosoma congolense* and *Trypanosoma vivax* infections in Cattle. *Veterinary Parasitology* **79**, 187–201.

FERGUSON, M. A., HOMANS, S. W., DWEK, R. A. & RADEMACHER, T. W. (1988). The glycosyl-phosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoprotein. *Biochemical Society Transactions* **16**, 265–268.

GARDINER, P. R., NENE, V., BARRY, M., THATTHI, R., BURLEIGH, B. & CLARKE, M. (1996). Characterization of a small variable surface glycoprotein from *Trypanosoma vivax*. *Molecular and Biochemical Parasitology* **82**, 1–11.

GRAB, D. J. & BWAYO, J. J. (1982). Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*. *Acta Tropica* **39**, 363–366.

HOLDER, A. A. (1985). Glycosylation of the variant surface antigens of *Trypanosoma brucei*. *Current Topics in Microbiology and Immunology* **117**, 57–74.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature, London* **227**, 680–685.

LANHAM, S. M. & GODFREY, D. G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Experimental Parasitology* **28**, 521–534.

LEFRANÇOIS, T., SOLANO, P., DE LA ROCQUE, S., BENGALY, Z., REIFENBERG, J. M., KABORE, I. & CUISANCE, D. (1998). New epidemiological features on animal trypanosomiasis by molecular analysis in the pastoral zone of Sideradougou, Burkina Faso. *Molecular Ecology* **7**, 897–904.

MAJIWA, P. A. O., THATTHI, R., MOLOO, S. K., NYEKE, J. H. P., OTIENO, L. H. & MALOO, S. (1994). Detection of trypanosome infections in the saliva of tsetse and buffy-coat samples from antigenaemic but aparastitaemic cattle. *Parasitology* **108**, 1–10.

MASIGA, D. K., SMYTH, A. J., HAYES, P., BROMIDGE, T. J. & GIBSON, W. C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology* **22**, 909–918.

MOSER, D. R., COOK, G. A., OCHS, D. E., BAILEY, C. P., MCKANE, M. R. & DONELSON, J. E. (1989). Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using the Polymerase Chain Reaction. *Parasitology* **99**, 57–66.

MURRAY, M., MURRAY, P. K. & MCINTYRE, W. I. (1977). An improved parasitological technique for diagnosis of African Trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **71**, 325–326.

PEREGRINE, A. S. (1994). Chemotherapy and delivery systems: haemoparasites. *Veterinary Parasitology* **54**, 223–248.

REBESKI, D. E., WINGER, E. M., ROBINSON, M. M., GABLER, C. M. G., DWINGERM, R. H. & CROWTHER, J. R. (2000). Evaluation of antigen-coating procedures of enzyme-linked immunosorbent assay method for detection of trypanosomal antibodies. *Veterinary Parasitology* **90**, 1–13.

REBESKI, D. E., WINGER, E. M., VAN ROOIJ, E. M., SCHOCHL, R., SCHULLER, W., DWINGER, R. H., CROWTHER, J. R. & WRIGHT, P. (1999). Pitfalls in the applications of enzyme-linked immunoassays for the detection of circulating trypanosomal antigens in serum samples. *Parasitology Research* **85**, 550–556.

REIFENBERG, J. M., SOLANO, P., DUVALLET, G., CUISANCE, D., SIMPORE, J. & CUNY, G. (1997). Molecular characterization of trypanosome isolates from naturally infected domestic animals in Burkina, Faso. *Veterinary Parasitology* **71**, 251–262.

REYNA-BELLO, A., GARCIA, F., RIVERA, M., SANSÓ, B. & ASO, P. (1998). Enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Trypanosoma evansi* equine antibodies. *Veterinary Parasitology* **1483**, 1–9.

RAUSCH, S., SHAYAN, P., SALNIKOFF, J. & REINWALD, E. (1994). Sequence determination of three variable surface glycoproteins from *Trypanosoma congolense*. Conserved sequence and structural motifs. *European Journal of Biochemistry* **223**, 813–821.

SCHNEIDER, P., FERGUSON, M. A. J., MCCONVILLE, M. J., MEHLERT, A., HOMANS, S. W. & BORDIER, C. (1990). Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. *Journal of Biological Chemistry* **265**, 16955–16964.

SEIDL, A., DÁVILA, A. M. & SILVA, R. A. (1999). Estimated financial impact of *Trypanosoma vivax* on the Brazilian pantanal and Bolivian lowlands. *Memórias do Instituto Oswaldo Cruz* **94**, 269–272.

SHAW, J. & LAINSON, R. (1972). *Trypanosoma vivax* in Brazil. *Annals of Tropical Medicine and Parasitology* **66**, 25–32.

SOLANO, P. & AMSLER-DELAFOSSE, S. (1995). *Trypanosoma congolense* in different species of horse flies (Diptera: Tabanidae) in Burkina Faso. *Revue Élevage et de Medecine Veterinaire des Pays Tropicaux* **48**, 145–146.

STERVERDING, D. & KREMP, K. (1998). An improved method for the purification of *Trypanosoma brucei* variant surface glycoprotein. *Parasitology Research* **84**, 524–525.

TORO, M., LEÓN, E., LÓPEZ, R. & RUÍZ, A. (1980). Resultados de un muestreo sobre tripanosomiasis bovina mediante técnicas serológicas. *Veterinaria Tropical* **5**, 43–50.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76**, 4350–4354.

TUNG, A. S., JU, S. T., SATO, S. & NISONOFF, A. (1976). Production of large amounts of antibodies in individual mice. *Journal of Immunology* **116**, 676–681.

TURNER, M. J., CARDOSO DE ALMEIDA, M. L., GURNETT, A. M., RAPER, J. & WARD, J. (1985). Biosynthesis, attachment and release of variant surface glycoproteins of the African trypanosome. *Current Topics in Microbiology and Immunology* **117**, 23–55.

UCHE, U. E., ROSS, C. A. & JONES, T. W. (1992).

Identification of the surface components of *Trypanosoma evansi*. *Research in Veterinary Science* **53**, 252–253.

UCHE, U. E., JONES, T. W. & BOID, R. (1993). Class-specific antibody response in rabbits experimentally infected with *Trypanosoma evansi*. *Tropical Medicine and Parasitology* **44**, 27–31.

VICKERMAN, K. (1969). On the surface coat and flagellar adhesion in trypanosomes. *Journal of Cell Science* **5**, 163–193.

WELLS, E. A. (1984). Animal trypanosomiasis in South America. *Preventive Veterinary Medicine* **2**, 31–41.

WOO, P. T. K. (1970). Haematocrit centrifuge detective of trypanosomes in blood. *Acta Tropica* **27**, 384–386.

WOOD, J. G. & SARINANA, F. O. (1975). The staining of sciatic nerve glycoproteins on polyacrylamide gels with concanavalin A-peroxidase. *Annals of Biochemistry* **69**, 320–322.

ZAMZE, S. E., FERGUSON, M. A. J., COLLINS, R., DWEK, R. A. & RADEMACHER, T. W. (1988). Characterization of the cross-reacting determinant (CRD) of the glycosyl-phosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoprotein. *European Journal of Biochemistry* **176**, 527–534.