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The Cytostome of *Trypanosoma cruzi* Epimastigotes Is Associated with the Flagellar Complex

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Okuda, K., Esteva, M., Segura, E. L., and Bijovsky, A. T. 1999. The cytostome of *Trypanosoma cruzi* epimastigotes is associated with the flagellar complex. *Experimental Parasitology* **92,** 223–231. Proliferative forms of *Trypanosoma cruzi*, amastigotes and epimastigotes, have a cytostome, a specialized structure formed by an invagination of the flagellar pocket's membrane surrounded by microtubules and frequently followed by a row of vesicles. All this assemblage penetrates deeply into the cytoplasm overpassing the nucleus. This structure, together with the flagellar pocket, appears to play an important role in the nutrition of the parasite. We demonstrated that the monoclonal antibody 2C4, made-up against isolated flagellar complex of *T. cruzi* epimastigotes, recognizes a protein doublet of 76 and 87 kDa in total epimastigotes homogenate. The 76-kDa polypeptide is enriched in the detergent-soluble fraction whereas the 87-kDa polypeptide is highly represented in the insoluble fractions and the purified flagella. Immunofluorescence assays show the antigen as a small spot at the flagellar pocket region. Immunogold labeling of ultrathin sections of epimastigote forms reveals gold particles at the opening of flagellar pocket, concentrated in the cytostome region. Immunocytochemistry of epimastigote whole-mount cytoskeletons reveals the labeling on an array of three to four microtubules that appears attached to flagellum, running in the direction of the nucleus. Ultrastructural observations have shown that the posterior region of isolated flagella, corresponding to the level of the flagellar pocket, possesses a microtubular structure compatible with that from the cytostome. The relationship between the cytostome, an endocytic organelle, and the flagellum is here described for the first time.

Index Descriptors and Abbreviations: Trypanosoma cruzi; epimastigotes; flagellar complex; cytostome; immunocytochemistry; ultrastructure.

INTRODUCTION

Trypanosoma cruzi is a flagellate protozoan parasite which, as the other members of the Trypanosomatidae family, possesses only one cytoskeletal structure: a microtubular array that involves the protozoan cell body and constitutes a membrane skeleton (Angelopoulos 1970). This cytoskeleton is present throughout all of the cell cycle (Sherwin and Gull 1989) and must be responsible for the different forms and shapes that these protozoa assume during their cell cycle. Until now, no transcellular cytoskeletal structures, such as intermediate filaments or microfilaments, have been identified despite actin having been described in *T. cruzi* (Mortara 1989) and actin mRNA in *T. brucei* (Ben Amar *et al.* 1988).

T. cruzi possesses a very complex cell cycle, with two extracellular forms, epimastigote and trypomastigote, and one intracellular stage, the amastigote. Amastigotes have only a short intracellular flagellum, which ends at the flagellar pocket opening. Both extracellular forms have a flagellum that, besides its main motility function, also has an important role in parasite adhesion to different cell and surface types (Vickerman and Tetley 1990).

Trypanosomatid flagellum arises from a basal body located anteriorly and orthogonally to the kinetoplast (Vickerman and Preston 1976; De Souza and Souto-Padron 1980). The extracellular flagellum goes further from the cell body through the flagellar pocket. This is a unique compartment, formed by an invagination of the plasma membrane, which constitutes the cellular site for endo- and exocytosis of macromolecules (Webster and Russell 1993). After exiting the

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cell, the flagellum remains adhered to the cell body up to the anterior end of the cell, where it becomes free. Extracellular flagella contain two major cytoskeletal structures: the classic 9 + 2 axoneme and a conspicuous fibrillar structure, the paraxial or paraflagellar rod (Vickerman and Preston 1976; De Souza and Souto-Padron 1980; Cachon *et al.* 1988).

Axonemal microtubules are more stable to detergent treatment than those of the membrane skeleton. However, both contain the same α-tubulin isoforms (Schneider *et al.* 1987). Furthermore, both subsets of microtubules appear to support the same post translational modifications of α-tubulin, acetylation and detyrosination (Sasse and Gull 1988) and an extensive glutamylation (Schneider *et al.* 1997). Despite the morphological description of dynein arms attached to the axonemal microtubule doublets (Souto-Padron *et al.* 1984; Hemphill *et al.* 1991), no convincing biochemical evidence for the existence of that protein has been disclosed.

Four particular microtubules of the subpellicular array are also resistant to detergent extraction and remain attached to isolated flagella. Sherwin and Gull (1989) suggested that the association between these particular microtubules with the flagellum would be responsible for the flagellar pocket topography. Various filamentous proteins were described associated with the microtubule quartet and involved in the flagellar–cell body adhesion (Cotrim *et al.* 1995; Ruiz-Moreno *et al.* 1995).

In addition to the locomotive function, the flagellum is an important structure of adhesion to cellular or acellular substrates (Tetley and Vickerman 1985; Hendry and Vickerman 1988; Beattie and Gull 1997). Moreover, flagellum plays an important role in the penetration of mammalian cells (Hemphill *et al.* 1991). Arrangement and length of the free flagellum were also related to important events of cytoskeletal morphogenesis along the *T. brucei* cell cycle (Sherwin and Gull 1989; Robinson and Gull 1991; Robinson *et al.* 1995).

It is also known that epimastigote flagella are involved in antigenic activity. Human chagasic patients display antibodies to major structures of *T. cruzi* flagellum. It has been shown that nearly 10% of the antigenic activity of the epimastigotes employed for antibodies search is localized in the flagellum (Piras *et al.* 1981). Furthermore, experimental immunoprotection in Chagas' disease has been described using flagellar fraction of epimastigotes (Segura *et al.* 1977; Ruiz *et al.* 1985, 1986; Wrightsman *et al.* 1995; Hansen *et al.* 1996; Miller *et al.* 1996, 1997).

Bearing in mind the biological and immunological importance of the flagellar fraction, the characterization of the antigen recognized by the monoclonal antibody (mab) 2C4, constructed against epimastigote flagellar fraction (Segura *et al.* 1986), has been undertaken.

These studies led us to recognize the cytostome in a microtubular structure that remains associated with the flagellum after the detergent extraction. As far as we know, this is the first time that an endocytic organelle, the cytostome, has been described as physically connected with the locomotive apparatus of *T. cruzi*.

MATERIAL AND METHODS

Parasites

The following organisms were included in this study. Epimastigote forms of *Blastocrithidia culicis*, *Trypanosoma conorhini*, and *T. cruzi*, CL strain, were cultured in liver infusion–tryptose (LIT medium) (Castellani *et al.* 1967) plus 10% bovine serum at 28°C. Tissue culture-derived trypomastigotes and amastigotes of *T. cruzi* were obtained from infected LLC-MK_2 cells (Andrews and Colli 1982). Promastigotes of *Leishmania (leishmania) amazonensis* were cultured in DL-15 medium (Pral *et al.* 1993).

Antibodies

Monoclonal antibody (mab) 2C4 was prepared as previously described (Segura *et al.* 1986). Briefly, BALB/c mice were injected with 200 μg/mouse of lyophilized flagellar fraction of epimastigotes (Tulahuen strain). Inactivated *Bordetella pertussis* (1.25×10^9 O.U.) was employed as adjuvant. Spleen cells were fused with myeloma P3-X63-Ag 8563 (Jk Ag8) cells. Tissue culture supernatants were screened for mab specific activity and limiting dilution cloned the hybrids.

Monoclonal anti-α-tubulin (Sigma Chemical Co.) and mouse anti-whole *T. cruzi* cytoskeleton sera were used as positive control. A nonrelated mab anti-*Plasmodium falciparum* was used as negative control.

Cytoskeleton Extraction

Protozoa cells were washed in RPMI medium by centrifugation, and incubated on ice in extraction medium: 1% Nonidet-P40 (NP-40) in PEME buffer (100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM Mg_2SO_4, 0.1 mM EDTA), containing 30% glycerol and a cocktail of protease inhibitors: 50 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml chymostatin, 5 μg/ml antipain, and 5 μg/ml phenylmethylsulfonyl fluoride (PMSF) (modified from Robinson *et al.* 1991). The lysates were examined by phase-contrast microscopy to check for the integrity of the cytoskeletons.

Isolation of Flagella

T. cruzi epimastigotes were collected and washed as described above, incubated for 15 min in 1% NP-40 in PBS at 4°C, centrifuged for 10

min at 1000*g*, and homogenized for 50 s with an "Omni uH Micro Homogenizer" (Omni International Inc.) using a Flat Bottom Generator Probe (G5-95) with pulses of 30,000 rpm. Homogenate was settled on a 0.6 M sucrose cushion and centrifuged for 15 min at 8000*g*. The pellet was collected and stored at −70°C until use. Negatively stained samples (see below) were always examined by electron microscopy to check the purity of the fraction.

Polyacrylamide Gel Electrophoresis and Western Blotting

Detergent lysates were centrifuged at 9000*g* for 15 min at 4°C, in order to obtain the soluble and insoluble fractions. The fractions were diluted in sample buffer, submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a 7% gel according to Laemmli (1970), and transferred to nitrocellulose membranes as described by Towbin *et al.* (1979). Antigenic polypeptides were developed by incubating membranes with the primary antibodies overnight at 4°C and, in sequence, for 2 h at 22°C with goat anti-mouse IgG labeled with peroxidase (Sigma Chemical Co.). Molecular weight markers were run in parallel: rabbit muscle myosin, *E. coli* β-galactosidase, rabbit muscle phosphorylase B, bovine serum albumin, chicken egg ovoalbumin, and bovine pancreas trypsinogen (Sigma Chemical Co.).

Immunofluorescence

Cytoskeletons of all trypanosomatids described above, obtained as described, were settled onto slides coated with 0.1% poly-L-lysine (Sigma Chemical Co.). Slides were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 30 min at 4°C. Slides were incubated with the primary antibodies for 30 min at 37°C in a moist atmosphere and washed in PBS. In sequence, the slides were incubated for 30 min with fluorescein-conjugated anti-mouse IgG (Sigma Chemical Co.), washed with PBS, and mounted with buffered glycerol (pH 7.2) containing 2.5 mg/ml sodium azide as antifading agent.

Electron Microscopy

Cytoskeleton preparations. Epimastigote forms of *T. cruzi* were washed in RPMI medium and allowed to settle onto Formvar-coated nickel grids before treatment with the extraction medium described above. For morphologic studies, cytoskeletons (CSK) were fixed in 2.5% glutaraldehyde in PEME and negatively stained with 2% ammonium molybdate, pH 6.9. The grids were observed in a JEOL 100 CX electron microscope.

Immunogold labeling of cytoskeletons. Cytoskeletons extracted as described above were fixed in 3.7% paraformaldehyde, 0.01% glutaraldehyde in PEME and washed in 20 mM glycine in phosphate-buffered saline. After blocking with 1% BSA (bovine serum albumin) in PBS, cytoskeletons were incubated in the first antibody at 22°C for 60 min, washed in 1% BSA/PBS, and incubated in the second antibody (goat anti-mouse 15-nm gold conjugate; Amersham, UK) diluted in 1% BSA/PBS for 1 h. After intensive washing of the grids in 1% BSA/PBS (2× 5 min), 0.1% BSA/PBS (2× 5 min), and PEME (2× 5 min), fixation was done in 2.5% glutaraldehyde in PEME for 15 min. Following fixation, the grids were negatively stained using 2% ammonium molybdate, pH 6.9.

Immunogold labeling of ultrathin sections. Epimastigotes were harvested, washed in RPMI medium, and fixed for 60 min in 4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% picric acid in 100 mM cacodylate buffer containing 1 mM $CaCl_2$ (pH 7.2). Cells were pelleted (10 min, 15,000*g*) and the pellets were dehydrated and infiltrated with LR White resin. Thin sections were mounted on nickel grids, rehydrated with H_2O and PBS, and incubated as were the cytoskeletons with the difference that the first incubation was done at 4°C, overnight. After postfixation, the grids were stained with uranyl acetate and lead citrate. Ultrathin sections of epimastigotes conventionally fixed and embedded in Spurr's resin were stained with uranyl acetate and lead citrate and studied as described above.

RESULTS

Western Blotting

2C4 Mab recognizes a doublet of proteins of approximate 76–87 kDa in total homogenate of *T. cruzi* epimastigotes (Fig. 1, lane A). When detergent-extracted, the 76-kDa polypeptide remains in the soluble fraction, whereas the insoluble fraction presents the 87-kDa polypeptide (Fig. 1, lanes B and C). The 87-kDa polypeptide is also present in isolated flagellar fraction (Fig. 1, lane D). The Mab did not recognize both *T. cruzi* trypomastigotes and amastigotes nor the other trypanosomatids tested (*T. conorhini* and *B. culicis* epimastigotes).

Indirect Immunofluorescence

When used in immunofluorescence assays of detergent-extracted *T. cruzi* epimastigotes, 2C4 Mab detects a small dot at the flagellar pocket region (Fig. 2). Consistent with the results obtained by Western blotting, the monoclonal antibody failed to recognize any structure in the other trypanosomatids investigated.

Immunogold Electron Microscopy

Whole-mount epimastigote cytoskeletons were harder to obtain through detergent extraction than those from trypomastigotes. Notwithstanding, with the addition of glycerol to the extraction solution (Heuser and Kirschner 1980), preparations retaining the original cell shape and form could be obtained. As described for other trypanosomatids (Angelopoulos 1970; Sherwin and Gull 1989), the major component of cell body cytoskeletons is a helical array of microtubules. Remnants of nuclei and kinetoplasts are retained in

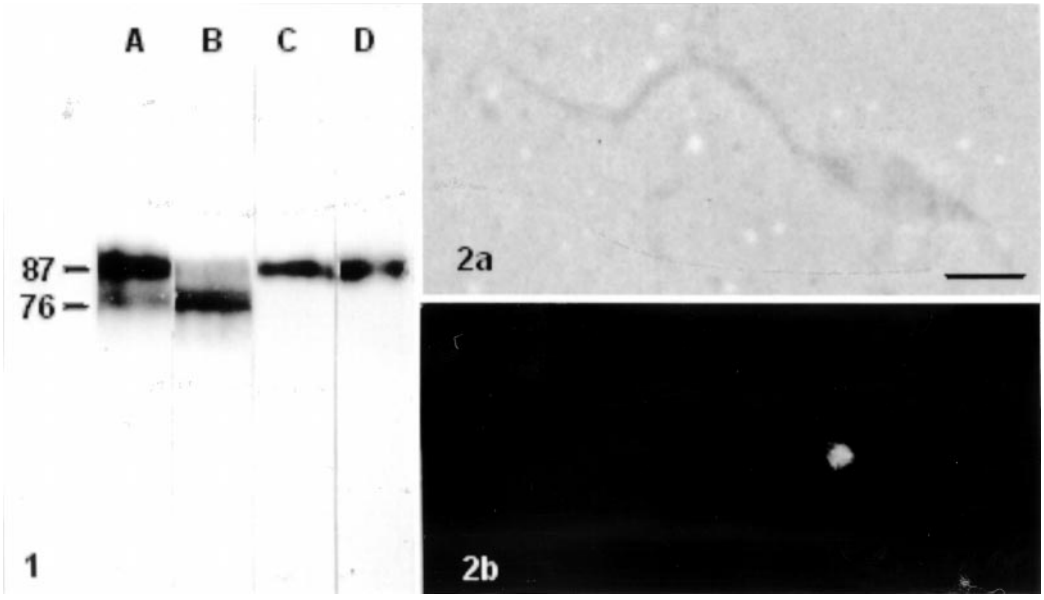

FIG. 1. Western blotting of *T. cruzi* epimastigotes. Mab 2C4 recognizes a doublet of 76–87 kDa in the total homogenate (lane A), a single polypeptide of 76 kDa in the NP-40 soluble fraction (lane B), and only the 87-kDa polypeptide in the insoluble fraction (lane C) and in isolated flagella (lane D).

FIG. 2. Phase-contrast (2a) and indirect immunofluorescence (2b) of a *T. cruzi* epimastigote cytoskeleton. It is possible to observe the label on the flagellar pocket region. Bar, 10.0 μm.

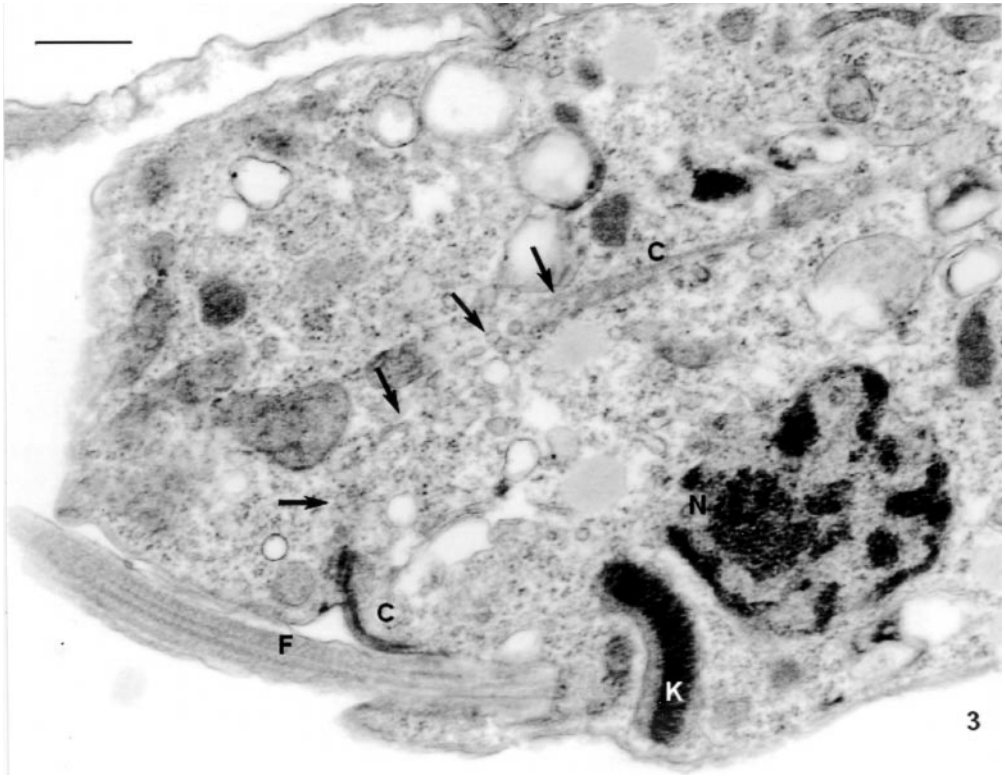

FIG. 3. Longitudinal section of a Spurr-embedded epimastigote. The cytostome (C) arises at the flagellar pocket and penetrates deeply into the cytoplasm. Vesicles are visualized along this structure (arrows). N, nucleus; K, kinetoplast. Bar, 0.5 μm.

their original cellular position. Flagellum contains the axoneme and the paraxial rod. In addition, epimastigote cytoskeletons retain an array of three to four microtubules that appears attached to flagellum, running in the direction of the nucleus (Fig. 4). This array of microtubules, which strongly resembles the cytostome, appears always attached to isolated flagella (Fig. 7).

In ultrathin sections of Spurr-embedded epimastigotes, cytostome always appears beginning at the bottom of the flagellar pocket or close to it, at the anterior portion of the cell (Fig. 3). It is a narrow cylindrical structure formed by an invagination of the cell membrane and by an array of four reflected subpellicular microtubules that run parallel to the whole cytostome. The organelle, which is almost always surrounded by vesicles, may penetrate deeply into the cytoplasm, overpassing the nucleus. Amorphous material is always present in the lumen of the organelle as well as inside the pocket.

Immunogold electron microscopy of epimastigote cytoskeletons reveals that the antigen detected by 2C4 Mab is located along the three to four microtubular structure of the cytostome, mainly at its proximal end (Fig. 4).

Immunogold labeling of ultrathin sections of epimastigote forms reveals gold particles at three defined locations: at the proximal portion of the cytostome tube (Figs. 5 and 6), at one side of the flagellar pocket (Figs. 5 and 6), and on the amorphous material present in the flagellar pocket or close to it (Fig. 5). Despite the fact that the 87-kDa polypeptide appeared enriched in the Western blotting of isolated flagella, we failed to label this structure in electron microscopy preparations of flagellar complex, probably due to the severe methods employed for the flagellum extraction.

DISCUSSION

Using a monoclonal antibody constructed against isolated flagella of epimastigote forms, the presence of a doublet of polypeptides (76–87 kDa) in the total lysate of epimastigote forms has been detected. When parasites were fractionated into detergent-insoluble cytoskeletons and detergent-soluble supernatant, we detected that the 76-kDa polypeptide is present in the soluble fraction whereas the cytoskeletal fraction

FIG. 4. Epimastigote whole-mount cytoskeleton. It is possible to observe the cytostome (C) through subpellicular microtubules. Cytostome begins at the proximal region of flagella (F) and internalizes toward the nucleus (N). Immunolabeling on the cytostome is concentrated at the flagellum proximity (arrowheads). K, kinetoplast. Bar, 0.5 μm.

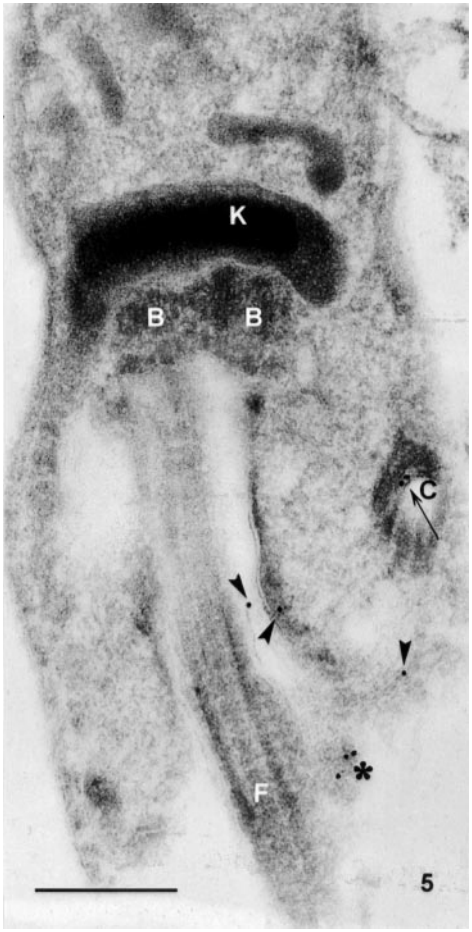

FIG. 5. Ultrathin section of a LR White-embedded epimastigote: immunogold labeling is present on one side of the flagellar pocket (arrowheads), inside the cytostome tube (arrow, C), and on amorphous material at the flagellar pocket opening (*). It is also possible to observe the elongate kinetoplast (K) and the duplicated basal body (B). F, flagellum. Bar, 0.5 μm.

contained the 87-kDa polypeptide, which was greatly enriched in the purified flagellar fraction.

The presence of the 87-kDa polypeptide could be demonstrated on a microtubular structure present in the epimastigote cytoskeleton that remained associated with flagella when they were isolated from the cell body. This microtubular structure was identified as the cytostome in epimastigote ultrathin sections processed by immunocytochemistry.

Proliferative forms of *T. cruzi*, amastigotes and epimastigotes, have a cytostome. This organelle, first described by Steinert and Novikoff (1960) in *T. mega* and by Milder and Deane (1969) in *T. cruzi* and *T. conorhini*, is a specialized structure formed by an invagination of the flagellar pocket's membrane, surrounded by microtubules and accompanied by numerous vesicles. This structure penetrates deeply into the cytoplasm toward the nucleus and, in association with the flagellar pocket membrane, appears to play an important role in the nutrition of the parasite. Indeed, it is known that, through both structures, the parasite ingests extracellular substances by pinocytosis (De Souza *et al.* 1978) and by receptor-mediated endocytosis (Soares and De Souza 1991).

Amorphous material is always present in the lumen of the cytostome (Milder and Deane 1969) and in the flagellar pocket. This amorphous material seems to be involved in trapping the substances to be ingested (Langreth and Balber 1975). Our observations always detected the presence of the antigen on the amorphous material inside or close to the cytostome opening, a fact that explains its presence in the detergent-soluble fraction.

The significance of the dual localization of this antigen and its possible secretion to extracellular media is not known. Studies investigating its potential secretion are currently being conducted in our laboratory. Other proteins, especially transporters, have been described as being located in both pellet and supernatant of *Leishmania*. The *myo*-inositol transporter of *L. donovani* (Drew *et al.* 1995; Snapp and Landfear 1997) and the two glucose transport isoforms of *L. enriettii* have different anchoring domains, both present at flagellar pocket and corresponding to different targeting signals for the flagellar or for the pellicular membrane (Snapp and Landfear 1997).

Another crucial question is why, although both the *T. cruzi* reproductive forms—amastigotes and epimastigotes—have a cytostome, mab 2C4 recognizes the 76- to 87-kDa protein in cytostome of *T. cruzi* epimastigotes only and not in cytostome of amastigotes. The principal function attributed to this structure is the uptake of nutrients. Both parasitic forms reside in different habitats. Epimastigotes live in the lumen of the invertebrate host and take their nutrients directly from the mammalian blood ingested by the bug. Soares and De Souza (1991) suggested that this could be the explanation for the fact that, in *T. cruzi*, receptor-mediated endocytosis is well developed only in the epimastigote form. Meyer and De Souza (1973) demonstrated that intracellular amastigotes could take cytoplasmic portions of the host cell through the cytostome. It is possible that these different methods of nutrient uptake could explain the antigenic differences observed herein.

The flagellar complex of trypomastigotes presents an array of four stable and specialized microtubules of the subpellicular array (SFMQ, subflagellar microtubule quartet; Gallo *et al.* 1987; Gallo and Precigout 1988). Our ultrastructural observations of isolated flagella of epimastigote show

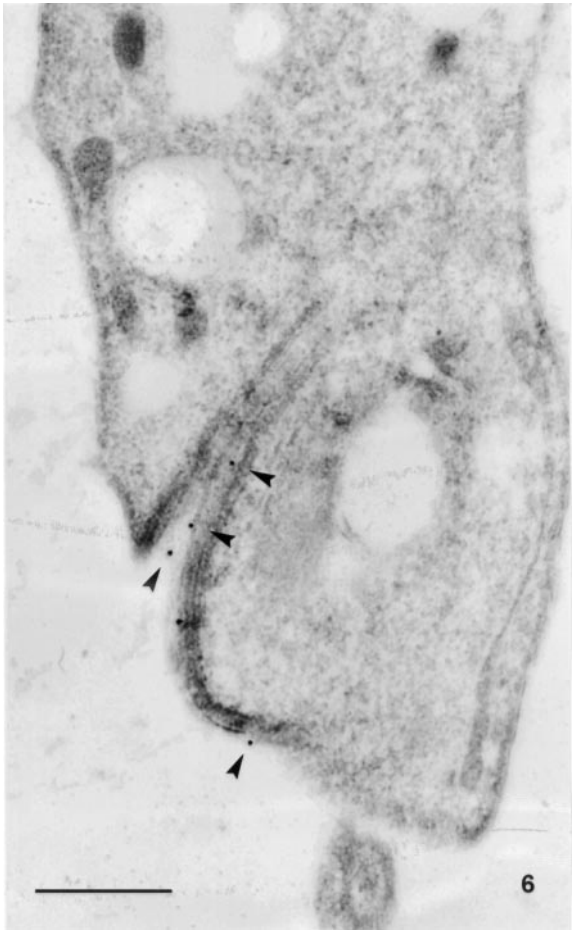

FIG. 6. Ultrathin section of an epimastigote form showing the labeling on the cytostome (arrowheads). Bar, 0.5 μm.

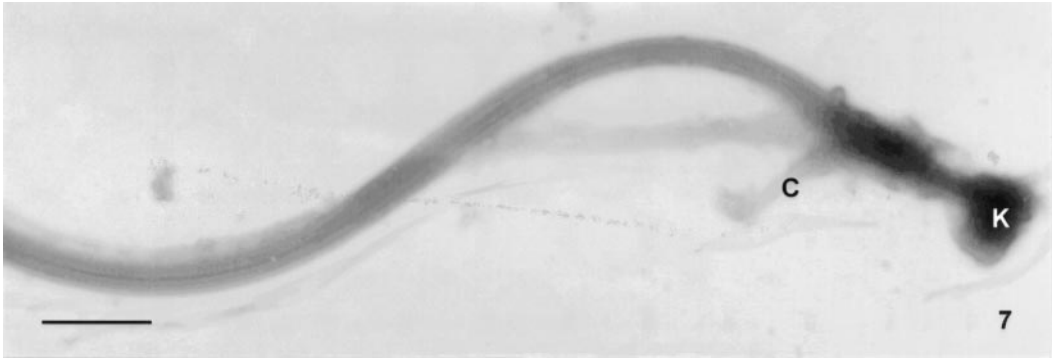

FIG. 7. Isolated flagella of *T. cruzi* epimastigote. Cytostome (C) arises near its proximal region. K, kinetoplast. Bar, 1.0 μm.

that the presence of SFMQ is not so constant as it is in trypomastigotes (Sherwin and Gull 1989; Ruiz-Moreno *et al.* 1995). In addition to this structure, the proximal region of epimastigote flagella have a microtubular structure consistent with the cytostome. This result would explain why the monoclonal antibody 2C4 (raised against isolated flagellar

complexes) recognizes the cytostome, which remains attached to the flagellar fraction after extraction. Association between cytostome and flagellum through basal body was already suggested in *T. lucknowi* by Weinman *et al.* (1984).

Souto-Padron *et al.* (1984) suggested a physical connection between the *T. cruzi* mitochondrion and the basal body that persists after detergent treatment. Robinson and Gull (1991) suggested that the structural and functional link between the kinetoplast, basal body, and flagellum is a mechanism to ensure position, replication, and segregation fidelity of these single-copy organelles throughout the cell cycle.

Due to the importance attributed to the cytostome, it is likely that physical link between this endocytic organelle and the flagellum also contributes to the segregation of this single-copy organelle.

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