Isolation and Characterization of Paraflagellar Proteins from
Trypanosoma cruzi*

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Two different Trypanosoma cruzi polypeptides, with masses of 70 and 68 kDa were purified and characterized in this work. These two polypeptides designated PAR 1 and PAR 2, respectively, co-purified during each step of the isolation procedure and were found to be located exclusively in T. cruzi flagella by indirect immunofluorescence. A pre-embedding immunoelectron microscopy procedure, with a gold-tagged secondary antibody, permitted direct identification of PAR 2 as a component of the T. cruzi paraflagellar rod. PAR 1 and PAR 2 were found to be immunologically distinct and showed no cross-reactivity with actin, tubulin, intermediate filament proteins, or other proteins present in mammalian cells. The results presented indicate that PAR 1 and PAR 2 are the major components of T. cruzi paraflagellar filaments, and that these filaments have no counterpart in mammalian cells.

Trypanosoma cruzi, a protozoan parasite, is the causative agent of American trypanosomiasis or Chagas’ disease (1). In Central and South America, this disease is an important public health problem (2), and adequate control is hampered by the lack of effective prophylactic or therapeutic agents. To date, considerable effort has been focused on parasite surface proteins in the belief that these molecules may provide prophylactic immunoprotection against the parasite (3-10). However, other molecules, structures, or cellular processes unique to T. cruzi which also may be suitable candidates for prophylactic or therapeutic intervention have received less attention. One such structure is the paraflagellar rod.

The paraflagellar rod is a major structural component of the T. cruzi flagellum and is identified as a complex lattice of filaments with ultrastructural characteristics unrelated to any of the major filamentous systems of eukaryotic cells, such as microfilaments, microtubules, or intermediate filaments (11). Proteins which are putative components of the paraflagellar filaments have been detected in several trypanosomatides (12-14). However, these proteins have not been shown by either biochemical or ultrastructural studies to be directly associated with the paraflagellar filaments. Since these proteins also have not been purified, it has not been possible to determine whether similar proteins are present in cells of the mammalian host. Likewise, no information is available on either the molecular organization of the paraflagellar filaments nor the mechanisms that regulate their assembly and breakdown of these structures during the morphological transformations which are an obligatory part of the life cycle of the parasite.

In this paper, we report on the purification and characterization of two T. cruzi polypeptides which are located exclusively in the flagellum of the parasite.

**MATERIALS AND METHODS**

**RESULTS**

Preparation of Monoclonal and Polyvalonal Antibodies against Flagellar-specific Proteins—The major structural components of T. cruzi flagella are the axonemic microtubules and the paraflagellar filaments (11). The microtubules also are found in the cell body of the parasite, as subpellicular components, while the paraflagellar filaments seem to be exclusively located in the flagella (11, 13). On this basis, hybridoma cell lines which produced monoclonal antibodies that identified antigens located exclusively in the flagellum were selected as reasonable candidates for production of anti-paraflagellar antibodies. Four such cell lines were identified by an indirect immunofluorescence assay. One of these hybridomas, designated hybPAR 2, also gave a positive reaction against a Western blot of total T. cruzi proteins and was chosen for further study.

That mAbPAR 2 reacts specifically with a flagellar antigen is indicated by two lines of evidence. First, in an indirect immunofluorescence assay, reaction of mAbPAR 2 with either epimastigotes (Fig. 1, panel a) or trypomastigotes (not shown) results in fluorescence being observed only over the flagellum. In contrast, antibodies against tubulin show fluorescence with both the flagellum and the cell body (Fig. 1, panel b). Second, in Western blots of total epimastigote lysates mAbPAR 2 reacts with a single polypeptide(s) with an apparent molecular weight of 68,000 (Fig. 2A, lane b), and this particular polypep-
FIG. 1. Immunostaining of T. cruzi epimastigotes. Coverslips with air-dried epimastigotes were fixed in acetone and incubated with pcAbPAR 1, mAbPAR 2, or anti-tubulin antibodies. The coverslips were next incubated with FITC-labeled goat anti-rabbit (pcAbPAR 1 and anti-tubulin samples) or FITC-labeled goat anti-mouse (mAbPAR 2 sample) antibodies. The cells were observed by epifluorescence microscopy. The different panels correspond to cells incubated with the following primary antibodies: a, mAbPAR 2; b, anti-tubulin; and c, pcAbPAR 1.

To further characterize the polypeptide(s) reactive with mAbPAR 2, epimastigote whole cell lysates and crude flagellar preparations were fractionated by two-dimensional gel electrophoresis. In a Western blot of a whole cell extract (Fig. 2B, panel b), mAbPAR 2 reacts with a single component of Mr = 68,000 and pI 6.5, thus strongly suggesting that this antigen is a single polypeptide. In two-dimensional gel profiles of epimastigote whole cell extracts stained with Coomassie Blue (Fig. 2B, panel a), this polypeptide, designated PAR 2, can be seen as a minor but clearly detectable component. It is enriched in a crude flagellar fraction (Fig. 2B, panel c), consistent with the results observed with the one-dimensional PAGE studies. Interestingly, examination of the two-dimensional gel profile of the flagellar fraction shows that a second polypeptide of apparent Mr = 70,000 and pI value slightly more acidic than that of PAR 2 is equally enriched in this fraction. The second polypeptide was designated PAR 1.

Collectively, these observations indicate that PAR 1 and PAR 2 are specifically located in the flagellum of T. cruzi, and that they are immunologically distinct. It is important to point out that since two different primary and two different secondary antibodies were used in these experiments the intensity of the bands or spots in the Western blots gave no quantitative indication of the relative mass of PAR 1 and PAR 2.

The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Bis-Tris, 2-[bis(2-hydroxyethyl) amino]-2-(hydroxyethyl)-propane-1,3-diol.
Probing of PAR 1 and 2 with Antibodies against Major Cytoskeletal Proteins—To determine whether PAR 1 and 2 are immunologically related to any of the major cytoskeletal proteins, Western blots of purified PAR 1 and PAR 2 were probed with antibodies against actin, tubulin, and intermediate filament proteins. A crude flagellar preparation and a rat brain extract that contains proteins recognized by those antibodies were included as controls in these experiments. As shown in Fig. 5, the anti-actin antibody reacts with the actin present in rat brain extracts (lane d), but does not react with purified PAR 1 and 2 (lane b) or with any polypeptide in the brain extract that contains proteins recognized by those antibodies were included as controls in these experiments. As shown in Fig. 5, the anti-actin antibody reacts with the actin present in rat brain extracts (lane d), but does not react with purified PAR 1 and 2 (lane b) or with any polypeptide in the brain extract that contains proteins recognized by those antibodies were included as controls in these experiments. As shown in Fig. 5, the anti-actin antibody reacts with the actin present in rat brain extracts (lane d), but does not react with purified PAR 1 and 2 (lane b) or with any polypeptide in the
crude flagellar preparation (lane c). The anti-tubulin antibody does not react with purified PAR 1 or PAR 2 (lane e), but does react with a polypeptide in the crude flagellar preparation (lane f) that has an electrophoretic mobility similar to that of β-tubulin from rat brain extracts (lane g). The antibody against intermediate filament proteins recognizes a polypeptide with \( M_r = 66,000 \) in a crude flagellar preparation (lane i), but it does not react with purified PAR 1 or 2 (lane h). As expected, mAbPAR 2 recognizes PAR 2 in both the preparation of purified protein (lane k) and the crude flagellar fraction (lane l), but does not react with any polypeptide in the rat brain extract (lane m). A similar result was obtained with pcAbPAR 1 (not shown).

**DISCUSSION**

Two *T. cruzi* polypeptides, PAR 1 and PAR 2, with apparent masses of 70 kDa and 68 kDa, respectively, were isolated and characterized in this study. The results of immunoelectron microscopy studies, with a gold-tagged secondary antibody, directly demonstrated that PAR 2 is a component of the paraflagellar rod in the *T. cruzi* flagellum (Fig. 4). Although direct localization of PAR 1 was not possible, the results of the immunofluorescence studies with pcAbPAR 1 showing that PAR 1 is localized in the *T. cruzi* flagellum and the observation that PAR 1 and PAR 2 both co-purify and are in approximately equimolar amounts suggest that PAR 1 also may be associated with the paraflagellar rod.

In the previous studies, two different polypeptides have been described as putative components of the paraflagellar rod. In *T. brucei*, these polypeptides have masses of 75 and 72 kDa (14), in *Cricthidia fasciculata* of 76 and 68 kDa (12), and in *Herpetomonas megaseliae* of 78 and 73 kDa (13), while in *Euglena gracilis*, a more distantly related organism, polypeptides with masses of 80 and 69 kDa (18) and of 76 and 67 kDa (14) have been detected. Gallo and Schevrel (14) reported that a monoclonal antibody against putative paraflagellar proteins reacted in Western blots with two *T. brucei* polypeptides (75 and 72 kDa) and concluded that a common epitope in these polypeptides was recognized by the monoclonal antibody. Our results, however, show that both the polyclonal antibodies against PAR 1 and monoclonal antibodies against PAR 2 react in two-dimensional Western blots with a distinctively different single polypeptide (Fig. 3B), which indicates a lack of antigenic cross-reactivity between PAR 1 and PAR 2. We have no explanation for the discrepancy between our results and those of Gallo and Schevrel (14). It is important to mention, however, that if the SH-dependent protease present in *T. cruzi* extracts is not completely inhibited, both of our antibodies react in Western blots with a variable number of degradation products of PAR 1 or PAR 2 (see Purification of PAR 1 and 2 in the Miniprint). In the case of PAR 1, one of these degradation products migrates in one-dimensional gels precisely in the position of PAR 2, a coincidence that could lead to the erroneous conclusion that PAR 1 and PAR 2 are immunologically related.

The results presented in Fig. 5 indicate that PAR 1 and PAR 2 are not immunologically related to any of the major cytoskeletal proteins of eukaryotic cells. Moreover, neither pcAbPAR 1 nor mAbPAR 2 react with any polypeptide in rat brain extracts or in any other mammalian cell so far analyzed. These results indicate that the paraflagellar protein PAR 2 and the putative paraflagellar protein PAR 1 may indeed be unique to the parasite, with no counterpart in the host cell. Two observations, however, indicate that the paraflagellar rod may contain other molecular components, some of which could also be present in the host cell. First, in Western blots, an antibody against intermediate filaments reacts with a 66-kDa polypeptide which is present in both a crude flagellar fraction (Fig. 5, lane i) and a rat brain extract (Fig. 5, lane j).

Whether the polypeptides in these two samples are identical and whether the one from *T. cruzi* is a component of the paraflagellar rod is not known. Secondly, detailed ultrastructural studies have shown that at least two different types of filaments, with diameters of 7 nm and 25 nm, form the complex lattice of the paraflagellar rod (19). It is conceivable that these two types of filaments share common polypeptides and represent different stages of aggregation. It is equally possible that the two types of filaments are composed of different polypeptides. Since the results of our immunoelectron microscopy studies do not allow us to determine the type of paraflagellar filament in which PAR 2 is located, we cannot distinguish between these two possibilities.

These results support a working model in which two different polypeptides, immunologically distinct, are the major components of *T. cruzi* paraflagellar filaments. These filaments are unique to the parasite, with no counterpart in the host cell. A compelling possibility is that a heterodimer, formed by PAR 1 and PAR 2, serves as a building block for the formation of paraflagellar filaments. This and other questions concerning the organization of the paraflagellar rod will be directly approachable once the conditions for the *in vitro* formation of paraflagellar filaments are established. Equally important is to develop immunoelectron microscopic procedures in which simultaneous preservation of antigen reactivity and optimal ultrastructural detail could allow unambiguous identification of the molecular components of the different types of paraflagellar filaments. These problems are currently being addressed.

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**REFERENCES**

T. cruzi Paralarflagellar Proteins

**EXPERIMENTAL MATERIAL:**

**PURIFICATION OF PAR 1 AND PAR 2:**

**Protein Extraction:**

**Protein Solubilization:**

**Protein Purification:**

**Protein Characterization:**

**Immunofluorescence and Immuno EM:**

**Electrophoresis and Western Blot Analysis:**

**Polyacrylamide Gel Electrophoresis (PAGE):**

**Densitometry:**

**References:**

**Figure 6:** Purification of PAR 1 and PAR 2.

**Figure 7:** Purification of PAR 1 and PAR 2 at neutral pH.

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*For image EM 7, trypanosomes were washed twice with and resuspended in PBS at a density of 10^7 parasites per ml. Aliquots (100 μl) of the cell suspension were pipetted onto 12 mm glass coverslips, which were kept at 4°C for 30 minutes. Special care was taken to prevent air-drying of the preparations during the following stages. The coverslips were transferred in solution to methanol and to 2% osmium tetroxide in PBS. The coverslips were then washed with PBS, were incubated for 30 minutes at room temperature with the primary antibody (anti-mouse IgG) containing 5% PBS (PBS-5%BSA) and 0.1% Tween 20. After rinsing with PBS, the preparations were incubated for 30 minutes with the secondary antibody, which was either labeled with FITC (Fluorescent Chemical Company) or labeled with 3,3'-diaminobenzidine peroxide (Jansen Life Science Products). The coverslips were rinsed with PBS, then washed with PBS-5%BSA, and observed with a fluorescence microscope.*