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Evidence for Four Distinct Major Protein Components in the Paraflagellar Rod of Trypanosoma cruzi*

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The major structural proteins present in the paraflagellar rod of Trypanosoma cruzi migrate on SDS-polyacrylamide gels as two distinct electrophoretic bands. The gene encoding a protein present in the faster migrating band, designated PAR 2, has been identified previously. Here we report the isolation and partial characterization of three genes, designated par 1, par 3, and par 4, that encode proteins present in the two paraflagellar rod protein bands. Peptide-specific polyclonal antibodies and monoclonal antibodies against the four proteins encoded by these genes shows that PAR 1 and PAR 3 are present only in the slower migrating paraflagellar rod band, and that PAR 2 and PAR 4 are present only in the faster migrating band. Analysis of the nucleotide sequence of these genes and the amino acid sequence of the conceptual proteins encoded by them indicates that par 2 shares high sequence similarity with par 3 and both are members of a common gene family, of which par 1 may be a distant member. Analysis of gene copy number and steady-state RNA levels suggest that the close stoichiometric ratio of the four PAR proteins is likely maintained by homeostatic regulation of RNA levels rather than gene dosage.

Trypanosoma cruzi, a hemoflagellate parasite, is the causative agent of American trypanosomiasis or Chagas' disease (1). This disease is prevalent throughout most of Central and South America, and no effective chemotherapeutic agent or immunoprophylactic intervention has been developed. The homologues of par 1, par 3, and both are members of a common gene family, of which par 1 may be a distant member. Analysis of gene copy number and steady-state RNA levels suggests that the close stoichiometric ratio of the four PAR proteins is likely maintained by homeostatic regulation of RNA levels rather than gene dosage.

Trypanosoma cruzi, Trypanosoma brucei, Leishmania amazonensis, and Leishmania mexicana are found in most of the length of the flagellum of Trypanosomatids and Euglenoids and dinoflagellates (2, 3). This unique structure is found only in these organisms, and its protein components are structurally and immunologically distinct from any of the major filamentous systems of the host cell, including microfilaments, microtubules, or intermediate filaments (4, 5).

A frequently used method of identifying promising targets for chemotherapeutic or immunoprophylactic intervention has focused on structures or cellular processes that are unique to the parasite. One such unique structure present in T. cruzi and almost all of the other members of the order Kinetoplastida is the paraflagellar or paraxial rod. The paraflagellar rod (PFR)1 is a complex lattice of filaments that runs parallel to the axoneme throughout most of the length of the flagellum of Trypanosomatids and Euglenoids and dinoflagellates (2, 3). This unique structure is found only in these organisms, and its protein components are structurally and immunologically distinct from any of the major filamentous systems of the host cell, including microfilaments, microtubules, or intermediate filaments (4, 5).

The major PFR proteins have been most extensively studied in the parasitic hemoflagellates T. cruzi, Trypanosoma brucei, Leishmania amazonensis, and Leishmania mexicana (Table 1). In these organisms, the major PFR proteins migrate in two bands on SDS-PAGE with electrophoretic mobilities of about 70–75 and 68–72 kDa and appear to be present in approximately equimolar amounts. The complexity of the proteins present in these two bands has been difficult to assess because classical biochemical techniques have not been highly successful in separating these proteins (6, 7). Additionally, most polyclonal or monoclonal antibodies against these proteins identify both bands, suggesting that the proteins present in these bands share common epitopes (4, 6, 8–10). These observations led to the suggestion that the two electrophoretic bands contained either a single protein that exhibits different conformations leading to different electrophoretic migration properties, or that the bands contained two different gene products that share amino acid (aa) similarity (4, 6, 7, 9).

This issue was apparently resolved by studies in T. brucei where genes coding for proteins present in both the slower and faster migrating PFR bands have been isolated and designated pfr A and pfr C, respectively (6, 11). The conceptual proteins encoded by these genes share high aa sequence similarity, thus supporting the concept that the PFR proteins are closely related. The homologues of pfr A have been isolated and characterized in both T. cruzi and L. mexicana and have been designated par 2 and par 4, respectively (7, 12). The proteins encoded by these genes all share high aa sequence similarity.

In contrast, we have reported that proteins present in the slower and faster migrating PFR bands could be partially separated by chromatographic techniques, and that amino acid sequence analysis of the proteins present in these two bands indicates the presence of two distinct polypeptides that share no apparent sequence similarity (7). The protein in the slower migrating band was designated PAR 1, and that in the faster migrating band was designated PAR 2. The amino acids of peptides derived from the protein preparations enriched in PAR 2 were identified in the protein sequence deduced from the nucleotide sequence of the par 2 gene. However, the aa sequences of several peptides present in the preparations enriched in PAR 1 could not be found in the PAR 2 preparations, suggesting the presence of a polypeptide in the slower migrating band that is distinct from PAR 2. Subsequent review of the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) AF004380, AF005193, AF005194, AF005195, AF045059, AF045060, and AF047923.

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PFR C sequence showed that only one of the PAR 1 peptides showed high sequence similarity with a region of PFR C, suggesting that a homologue of pfr C may be present in T. cruzi. However, the issue of the origin of the remaining putative PAR 1 polypeptides remained unresolved. Here, we report the isolation and partial characterization of the gene that encodes the PAR 1 protein. We also report the isolation of the T. cruzi homologue of pfr C, as well as the isolation of a gene, designated par 4, that encodes a previously unidentified polypeptide present in the faster migrating PFR protein band. These results show that the two PFR protein bands contain four distinct proteins, only two of which show high aa sequence similarity. These results also provide an explanation of why these four proteins are difficult to separate by common biochemical techniques and why some antibodies generated against the PFR proteins recognize only one of the two electroetcoric bands, while others recognize both.

### EXPERIMENTAL PROCEDURES

**Parasites—**T. cruzi Esmeraldo clone 3 strain was obtained from James Dvorak, National Institutes of Health, Bethesda, MD. Growth and maintenance of epimastigotes and tissue culture derived trypomastigotes are as described previously (7). T. brucei procyclics were a generous gift of Paul England, Johns Hopkins University, Baltimore, MD.

**PAGE and Immunoblot Analysis**—For analysis of whole cell lysates, parasites were harvested from culture media by centrifugation, washed twice with phosphate-buffered saline, and solubilized by direct addition of boiling 2% SDS with boiling continued for 5 min. These and all other samples were separated by one-dimensional PAGE and transferred to nitrocellulose by methods previously described (4), using a transblot cell (Bio-Rad) overnight at 150 mA. Filters probed with mouse sera or nitrocellulose by methods previously described (4), using a transblot cell. Samples were separated by one-dimensional PAGE and transferred to nitrocellulose by methods previously described (4), using a transblot cell (Bio-Rad) overnight at 150 mA. Filters probed with mouse sera or nitrocellulose by methods previously described (4), using a transblot cell.

**Northern Blot Analysis**—For analysis of whole cell lysates, parasites were harvested from culture media by centrifugation, washed twice with phosphate-buffered saline, and solubilized by direct addition of boiling 2% SDS with boiling continued for 5 min. These and all other samples were separated by one-dimensional PAGE and transferred to nitrocellulose by methods previously described (4), using a transblot cell (Bio-Rad) overnight at 150 mA. Filters probed with mouse sera or nitrocellulose by methods previously described (4), using a transblot cell.

**DNA Library Construction and Screening—**An epimastigote poly(A) RNA cDNA library was constructed in the phage λ ZAP Express vector using a cDNA synthesis system according to the manufacturer's instructions (Stratagene Inc., La Jolla, CA). A genomic DNA library was constructed in the λ ZAP Express vector using epimastigote nuclear DNA as described (13). The cDNA library was screened using mouse monoclonal antibodies directed against purified PFR proteins as described elsewhere (7), and positive phage clones were identified by use of the picoBlue nucleoscreening system (Stratagene). Positive phage clones were rescanned using mouse polyclonal antibodies directed against PAR 1 peptide 4 (pAbPep4) or monoclonal antibodies mAbPar2 (4), 2B7, or 8G5. The λ genomic DNA library was probed with a radiolabeled oligonucleotide consisting of nucleotides 1011–1031 (5′-CGACCGAGATGCTGTGAG-3′) of the T. cruzi DNA insert of pTccPar1b (accession no. AF047023). The inserts present in phages showing positive hybridization were characterized by restriction enzyme mapping and direct nucleotide sequence analysis.

**DNA Sequencing—**DNA sequence information was obtained by use of the dyeoxy chain-termination method (14). Fragments to be sequenced were sequenced directly from the excised pBlK-CMV phagemid. Oligonucleotide sequencing primers were obtained from Integrated DNA Technologies, Inc., Coralville, IA.

**Preparation of Monoclonal and Peptide-specific Polyclonal Antibodies—**A preparation of polyclonal antibody was injected into BALB/cByJ mice, and the spleens from animals with a positive reaction were used to obtain antibody-producing mouse hybridomas as described previously (15, 16). Fifteen hybridoma cell lines that produced antibodies against PAR proteins were initially screened by indirect immunofluorescence on Sepharose-Protein G columns as described previously (15). For polyclonal antibody production against PAR 1, the synthetic peptide H2N-CHYVENKEKVLKRN-COOH, the first 15 amino acids of PAR 1, was radiolabeled with [32P]dNTP using the Ambion random prime kit (Ambion Inc., Austin, TX) as recommended by the manufacturer. Synthetic oligonucleotides were radiolabeled with [γ-32P]ATP using T4 polynucleotide kinase (Amer sham Pharmacia Biotech) as described previously (16). Agarose gel electrophoresis of DNA, Southern transfer, prehybridization, hybridization, and filter washing were performed as described (17) with the following exceptions. Gels were 1% agarose, and the electrophoresis was 42 °C following hybridization with the end-labeled oligonucleotide. DNA was electrophoresed in a formaldehyde gel, blotted to nylon, cross-linked by UV irradiation, prehybridized, hybridized, and washed as described (19). Northern and Southern blots were imaged using a Molecular Dynamics PhosphorImager 445SI. Analysis of the digital images was conducted using the program ImageQuant (Molecular Dynamics, Inc.). All restriction enzymes were purchased from Life Technologies, Inc. and used as recommended.

**Immunofluorescence—**Indirect immunofluorescence with preparations of air-dried epimastigotes and trypomastigotes, fixed with acetone at room temperature, was done using FITC-labeled goat anti-mouse as the secondary antibody as described previously (20). **Construction and Expression of Recombinant PAR 1**—The production of recombinant PAR 2 in the baculovirus expression system was accomplished as described elsewhere (21). For production of PAR 1, the entire PAR 1 coding region was expressed in the baculovirus expression system by cloning a 1.8-kb region of pTcPar1a (GenBank accession no. AF004380) containing nucleotides 8–1824 into the shuttle vector pVL1393. The fragment was inserted into the BamHI/XbaI sites in the baculovirus expression system by cloning a 1.8-kb region of pTcPar1a (GenBank accession no. AF004380) containing nucleotides 8–1824 into the shuttle vector pVL1393. The fragment was inserted into the BamHI/XbaI sites in the baculovirus expression system by cloning a 1.8-kb region of pTcPar1a (GenBank accession no. AF004380) containing nucleotides 8–1824 into the shuttle vector pVL1393.
polylinker of the shuttle vector by generating a BamHI restriction enzyme site at nucleotides 7–12 and an XbaI restriction enzyme site at nucleotides 1824–1829 by using the following oligonucleotides as primers for polymerase chain reaction amplification of the par 1 gene:

- 5'GAAATAGGATCCGACAATGGCGGTTTAC-3' representing nucleotides 1–28
- 5'ATTTCCTTCTAGAGTACAC-3' representing nucleotides 1816–1836 of par 1.

For production of PAR 3 and PAR 4 in the baculovirus expression system, the entire T. cruzi cDNA inserts in pTccPar3a and pTccPar4b were excised from the recombinant phagemid DNAs with restriction enzymes BamHI and NotI, which cleave only within the polylinker sites of the phagemid vector. The excised inserts were cloned directly into the BamHI/NotI sites in the polylinker of the shuttle vector pVL1393. Production of recombinant baculovirus, production of recombinant proteins, and protein purification were as described elsewhere (21).

Antigen Preparation—PFR proteins were purified as described previously (4). Briefly, 10¹¹ Peru strain epimastigotes were harvested by centrifugation, washed in phosphate-buffered saline, and lysed in 0.1 M Tricine (pH 8.5) containing 1% Nonidet P-40. The pellet was extracted with high salt buffer consisting of 0.1 M Tricine (pH 8.5), 1 M NaCl, and 1% Triton X-100, using sonication. This crude flagellar pellet was successively extracted with 2.0 and 6.0 M urea in 10 mM Tricine (pH 8.5). The resulting supernatant contains approximately 50% PFR and 50% tubulin. The PFR proteins were separated by SDS-PAGE on a Bio-Rad Prep Cell model 491. Fractions containing the PFR proteins were extensively dialyzed against phosphate-buffered saline, concentrated by centrifugation in a Centricon concentrator (Amicon, Beverly, MA) and sterilized by 0.22-μm filtration.

**RESULTS**

Isolation of the par 1, par 3, and par 4 Genes—The amino acid sequence of several peptides generated by CNBr cleavage of a protein(s) preparation highly enriched for protein(s) present in the slower migrating band of the PFR protein doublet have been reported (7). The protein that was the putative origin of these peptides was designated PAR 1. In order to identify the gene that might encode PAR 1, one of these peptides, peptide 4, was synthesized, coupled to ovalbumin, and used as an antigen for generating peptide-specific antisera in mice. As shown in Fig. 1, sera (pcAbPep4) from mice immunized with the peptide ovalbumin conjugate contain antibodies that recognize the slower migrating protein band but not recognize the faster migrating band, consistent with the belief that PAR 1 is present only in the slower migrating PFR band.

To isolate a cDNA fragment that contains at least a portion of the par 1 gene, an epimastigote cDNA expression library was constructed in λ ZAP and screened with polyclonal mouse serum directed against proteins present in both the slower and faster protein bands (7). Approximately 200,000 recombinant phage were screened, and 18 primary positive plaques were identified. Two plaques, designated λTccPar1a and λTccPar1b, were rescreened positive with pcAbPep4. The phagemids containing the T. cruzi DNA inserts were excised for further analysis. Restriction enzyme mapping analysis of the cDNA inserts in...
these two phagemids, designated pTccPar1a and pTccPar1b, reveal fragments of 1.0 and 1.9 kb, respectively. The complete nucleotide sequence of each of these DNA inserts was determined. Analysis of the nucleotide sequence revealed that pTccPar1a is entirely contained within pTccPar1b, that both share a common open reading frame and that both contain the peptide 4 coding region. However, since the open reading frame in the largest cDNA insert, pTccPar1b, terminates in the 5’ synthetic EcoRI linker, it is likely that neither cDNA insert contains the entire coding region of the PAR 1 protein. Since T. cruzi genes lack intron sequences, it seemed reasonable that the complete sequence of the par 1 gene could be obtained by analysis of genomic DNA.

To obtain genomic DNA fragments that putatively contained the par 1 gene, an oligonucleotide whose sequence is present in pTccPar1b, but is not found in the previously identified PFR genes, was synthesized, radiolabeled, and used as a probe to screen a λ recombinant genomic DNA library. Three recombinant phage were rescreened positive following plaque purification. The phagemids in each of the three λ phage were excised, a partial restriction enzyme map of each T. cruzi DNA insert was generated (data not shown), and those DNA fragments that contained sequences complementary to the cDNA insert in pTccPar1b were determined by Southern blot analysis. A partial nucleotide sequence of one of the four DNA inserts, pTcgPar1a, was determined.

Analysis of the DNA sequence of the recombinant phagemids pTccPar1a, pTccPar1b, and pTcgPar1a shows that they encode part or all of the PAR 1 protein. The complete nucleotide sequence of the T. cruzi DNA insert in pTccPar1a and pTccPar1b is present in pTcgPar1a. With the exception of the poly(A) sequences on the 3’ terminus of the cDNA inserts, no nucleotide sequence heterogeneity was observed in an alignment of the 3 sequences (data not shown). The predicted amino acid sequence of the PAR 1 protein encoded by pTcgPar1a is shown in Fig. 2. This conceptual translation product has a predicted Mr of 68,173, consistent with the observed Mr of 70,000, and it contains the amino acid sequences of six of the seven polypeptides identified by direct amino acid analysis of protein in the slower migrating PFR protein band (7). Additionally, expression of this gene in the baculovirus system generates a polypeptide of approximately 70 kDa that is recognized on Western blots by pcAbPep4 (Fig. 1).

Interestingly, the peptide sequence that previously was identified in the slower migrating PFR band (Ref. 7; peptide 14) that is not present in either PAR 1 or PAR 2 is found in the predicted aa sequence of the PFR C protein of T. brucei (11), suggesting that a gene similar to the pfr c gene of T. brucei may be present in the genome of T. cruzi. In an attempt to isolate the putative T. cruzi homologue of the pfr c gene of T. brucei, the 18 recombinant phage identified in the screen of the T. cruzi cDNA library with anti-PFR mouse serum were re-screened with monoclonal antibody mAbPAR2, which is monospecific for PAR 2 (4). Thirteen phage re-screened positive, indicating they likely encoded PAR 2, while five phage re-screened negative. The phagemid in each of the five phage was excised and the nucleotide sequence of the T. cruzi DNA insert in each was determined. Of the five phagemids, two were identified as the previously isolated pTccPar1a and pTccPar1b and three contained nucleotide sequences not present in par 1. These three phagemids were designated pTccPar3a, pTccPar3b, and pTccPar3c. The length of the T. cruzi DNA insert in each, as determined by direct nucleotide sequence analysis, was 1977, 1980, and 2223 bp, respectively (accession nos. AF005194, AF005195, and AF005193, respectively). Each T. cruzi DNA insert contains a single long open reading frame of 590 aa that encodes a conceptual protein of Mr 68,753 and contains the sequence of peptide 14. No differences in the aa sequence of the conceptual proteins encoded by these three DNA inserts was observed.

To further investigate the possibility that the two PFR protein bands might contain proteins other than PAR 1, PAR 2, and PAR 3, a bank of hybridoma cell lines producing monoclonal antibodies against the PFR proteins was generated from mice immunized with purified PFR protein. Seven independent cell lines were identified and expanded for further analysis. The specificity of the monoclonal antibodies produced by these cell lines was determined by Western blot analysis using purified PFR protein, recombinant PAR 1, recombinant PAR 2, and recombinant PAR 3 protein (Table II). Monoclonal antibodies obtained from each of the hybridoma lines were found to recognize either one or both of the two PFR protein bands on Western blots. In addition, mAb6B3 recognized only recombinant PAR 1, mAb2A10, recognized only recombinant PAR 2, monoclonal antibodies, 1A10, 4C11, and 7C2, recognized both recombinant PAR 2 and recombinant PAR 3, and surprisingly, mAb2B7 and mAb8G5, failed to recognize any of the recombinant PAR proteins. The observation that both monoclonal antibodies 2B7 and 8G5 recognized parasite-derived PFR protein, but not the recombinant proteins, suggested the presence of a protein(s) in the purified PFR protein preparation that contains epitopes not found in PAR 1, PAR 2, or PAR 3. To examine this possibility, the recombinant T. cruzi cDNA -λ ZAP library was screened with monoclonal antibody 8G5. Three recombinant phage were identified. Each phage also reacted positive with monoclonal antibody 2B7, suggesting that these two monoclonal antibodies may recognize the same protein. The phagemids, designated pTccPar4a, pTccPar4b, and pTccPar4c, were excised, and the complete nucleotide sequence of the T. cruzi DNA inserts in each was determined (GenBank accession nos. AF045059, AF045060, and AF045061, respectively). The lengths of the insert DNAs were found to be 1977, 1980, and 2223 bp, respectively. Analysis of these three DNA sequences revealed the following. 1) Each DNA contains a single long open reading frame that encodes a conceptual protein of Mr 68,281. 2) The aa sequence of the proteins predicted by the nucleotide sequence of the 3 different DNA inserts are identical. 3) The insert in pTccPar4b contains 14 nucleotides on its 5’ terminus that correspond in sequence to the 3’ terminus of the T. cruzi mini-exon.

Relationship between the PFR Proteins from T. cruzi, T. brucei, and L. mexicana—At present, sequence data are available for seven PFR proteins: PFR A and PFR C from T. brucei (6, 11), PFR 2 from L. mexicana (12), and PAR 1, PAR 2, PAR 3, and PAR 4 from T. cruzi (this study; Ref. 7). In a direct alignment, PFR A, PFR C, PFR 2, PAR 2, and PAR 3 exhibit significant sequence identity throughout most of their length (Figs. 2 and 3A). As shown in Fig. 3B, a calculation of the sequence distances of these PFR proteins indicates that PFR A, PAR 2, and PAR 2 constitute one gene group, while PFR C and PAR 3 form a second gene group. Interestingly, the regions of highest sequence dissimilarity between the members of these two groups occur at the NH₂ and COOH termini. PFR-A, PFR-2, and PAR 2 share only 2/11 aa at the NH₂ terminus, while PFR-C and PAR 3 share only 3/9 aa at the NH₂ terminus and 4/27 aa at the COOH terminus. Also suggested is a distant relationship between these PFR proteins and PAR 1. The sequence relationship between these six proteins also is evidenced by the results of data base searches that show significant similarities with no proteins other than the PFR proteins. In contrast, PAR 4 shares no significant sequence similarity with the other PFR proteins (Figs. 2 and 3). This interpretation
### Alignment of paraflagellar rod protein sequences

The deduced amino acid sequences of the DNA for the paraflagellar rod genes of *T. cruzi*, *T. brucei*, and *L. mexicana* are shown. Boxed residues are those which fit the consensus sequence. Amino acid sequences in PAR 1 that have been verified by direct amino acid sequence analysis are indicated by an underline.

**FIG. 2** Complexity of the Paraflagellar Rod Proteins of T. cruzi

| Alignment of paraflagellar rod protein sequences. The deduced amino acid sequences of the DNA for the paraflagellar rod genes of *T. cruzi*, *T. brucei*, and *L. mexicana* are shown. Boxed residues are those which fit the consensus sequence. Amino acid sequences in PAR 1 that have been verified by direct amino acid sequence analysis are indicated by an underline. PAR 2, a 69-kDa protein of *T. cruzi* (7); PFR A, a 70-kDa... |
is consistent with the observation that data bases searches with PAR 4 did not identify the other PFR proteins. However, these searches did reveal a low level (about 25%) of sequence similarity between PAR 4 and myosin heavy chain. The similarity with the myosin heavy chain extends uniformly along the total length of PAR 4 with no clustered regions of high sequence similarity being evident. However, no biological significance can be ascribed to this finding at this time.

**Number and Expression of the par 1, par 3, and par 4 Genes—**

The copy number of the par 1, par 3, and par 4 gene sequences in the genome of *T. cruzi* were determined by methods described previously (16). Oligonucleotides containing regions of each gene that share no significant sequence identity with other PFR genes were synthesized and radiolabeled with 32P. The specificity of each oligonucleotide for its corresponding gene was confirmed by hybridization of the three gene-specific probes with Southern blots containing the entire par 1, par 2, par 3, and par 4 DNA sequences. Each gene probe hybridized only with its respective gene (data not shown). The par 1 gene probe was hybridized to a Southern blot containing epimastigote nuclear DNA digested with Hpa II (Fig. 4). Included on the Southern blot was pTcgPar1a DNA restricted with Hpa II in amounts equivalent to 1, 2, 4, 8, 16, and 32 copies per haploid genome. Strong hybridization of the probe was observed with a single genomic fragment of length 1.4 kb. Quantitation of the hybridization signal in the genomic and plasmid DNAs with a PhosphorImager indicates that the Hpa II fragment occurs about 10 times per haploid genome. The par 3 gene probe was hybridized to a Southern blot identical to that described above except that it contained pTccPar3a DNA restricted with Hpa II in amounts equivalent to 1, 2, 4, 8, 16, and 32 copies per haploid genome. Hybridization was observed with a single genomic

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| **Table II** | **Reactivity of polyclonal and monoclonal antibodies with Western blots of parasite-derived PFR protein and recombinant PAR proteins** | | | | |
| Polyclonal and monoclonal antibodies | Reactivity with PFR protein and recombinant PAR proteins | | | | |
| | Purified PFR protein | Recombinant PAR 1 | Recombinant PAR 2 | Recombinant PAR 3 | Recombinant PAR 4 |
| pcAbPep4 | + | – | – | + | + |
| pcAbPAR3 | + | + | – | – | – |
| mAbPAR2 | + | – | + | – | – |
| 1A10 | + | + | + | – | – |
| 2A10 | + | + | – | – | – |
| 2B7 | + | – | – | – | – |
| 4C11 | + | – | – | – | – |
| 6B3 | + | – | – | – | – |
| 7C2 | + | – | – | – | – |
| 8G5 | + | – | – | – | – |

*a* Denotes recognition of only one of the two PFR protein bands.

*b* Denotes recognition of both the slower and faster PFR protein bands.

![Complexity of the Paraflagellar Rod Proteins of T. cruzi](image)

**FIG. 2—continued**

1. T. cruzi (6); PFR 2, a 69-kDa protein of *L. mexicana* (12); PAR 3, a 69-kDa protein of *T. cruzi* (this study); PFR C, a 70-kDa protein of *T. brucei* (11); PAR 1, a 68-kDa protein of *T. cruzi* (this study); PAR 4, a 68-kDa protein of *T. cruzi* (this study).
DNA fragment of length 845 bp. Quantitation of the radioactivity in the genomic and plasmid DNA bands indicates approximately four copies of the par3 gene per haploid genome.

Hybridization of the par4 gene probe to a Southern blot containing genomic DNA and pTccPar4a DNA both digested with PvuII/PstI revealed a single band of length 760 bp. Quantitation of the radioactivity in the hybridization signals indicates that only 1–2 copies of the par4 gene are present per haploid genome.

To determine the developmental expression pattern of the par1, par3, and par4 genes, Northern blots containing trypomastigote and epimastigote poly(A)1 RNA were hybridized with 32P-labeled oligonucleotides specific to either pTcgPar1a, pTccPar3a, or pTccPar4a, respectively. As shown in Fig. 5, the par1, par3, and par4 gene probes hybridized to single RNA bands in both the trypomastigote and epimastigote lanes of approximate lengths 3.5, 2.2, and 2.5 kb, respectively. The intensity of the hybridization signals observed with the trypomastigote and epimastigote RNAs showed some variation. Although transcripts from the par3 and par4 genes are present in approximately equimolar amounts in the trypomastigote and epimastigote forms of the parasite, transcripts from the par1 gene appear to be more abundant in the epimastigotes than in the trypomastigotes.

**Cellular Localization of the PAR 1, PAR 3, and PAR 4 Proteins**—The subcellular location of the PAR 1, PAR 3, and PAR 4 proteins in T. cruzi was determined by an indirect immunofluorescence assay. As shown in Fig. 6A, reaction of mAb6B3 with acetone-fixed trypomastigotes resulted in fluorescence only over the flagellum, while antibodies against tubulin were distributed over both the cell body and the flagellum (Fig. 6B), thus confirming the localization of PAR 1 to the flagellum (4). To determine whether PAR 3 also is localized to the flagellum of the parasite, mouse polyclonal antibodies (pcAbPar3) were generated against a synthetic polypeptide containing aa sequences unique to PAR 3. The specificity of pcAbPar3 was confirmed by the observations that it recognizes only recombinant PAR 3 on Western blots containing recombinant PAR 1, recombinant PAR 2, recombinant PAR 3, and recombinant PAR 4 proteins and that it recognizes a single protein band of
apparent molecular mass 70 kDa on Western blots containing purified PFR protein (Fig. 1). Following incubation of pcAbPar3 with acetone-fixed trypomastigotes, fluorescence was observed only over the flagellum of the parasite (Fig. 6C). That PAR 4 also is localized to the flagellum of the parasite was revealed by the observation that incubation of acetone-fixed trypomastigotes with monoclonal antibody 6B3 and 8G5 resulted in fluorescence only over the flagellum (Fig. 6D).

Localization of PAR 1, PAR 2, PAR 3, and PAR 4 in the PFR Protein Doublet by Western Blot Analysis—Previous studies using a combination of biochemical and immunological techniques have unambiguously localized PAR 2 to the faster migrating PFR band (7). These studies also provide data that suggest PAR 1 is present in the slower migrating PFR band. To determine which of the two PFR protein bands contain PAR 1, PAR 3, and PAR 4, Western blots of PFR protein were reacted with different combinations of the PAR-specific antibodies. As shown in Fig. 7A, reaction of a Western blot of PFR protein with antibodies specific for PAR 1, PAR 3, and PAR 4 resulted in fluorescence only over the flagellum (Fig. 6D). The coverslips were next incubated with FITC-labeled goat anti-rabbit (tubulin) or FITC-goat anti-mouse (4).

In contrast, PAR 4-specific monoclonal antibody 8G5 or 2B7 (data not shown) showed no reactivity with the T. brucei lysate.

DISCUSSION

Our principle finding is that four distinct proteins are present in the two protein bands observed on SDS-PAGE of highly purified PFR proteins from T. cruzi. The gene encoding one of these proteins in T. cruzi, PAR 2, has been reported previously (7), and the genes that encode the other three proteins, PAR 1, PAR 3, and PAR 4, now have been cloned, sequenced, and partially characterized (Table III). Using monoclonal and polyclonal antibodies that are monospecific for each of the four PAR proteins, PAR 4 has been shown to be present in the slower migrating PFR protein band while PAR 2 and PAR 4 are present in the faster migrating PFR band. Of the four PAR proteins, only PAR 2 has been shown by immunoelectron microscopic analysis to be a component of the paraflagellar rod (4). However, three lines of evidence indicate that PAR 1, PAR 3, and PAR 4 also are components of the PFR. 1) PAR 1, PAR 3, and PAR 4 co-purify with PAR 2. 2) Immunofluorescence studies using antibodies specific to PAR 1, PAR 3, and PAR 4 clearly show that these proteins are localized to the flagellum of the parasite. 3) Ongoing studies to investigate the spatial relationship of the four PAR proteins by immunoelectron microscopic analysis have revealed that PAR 1 is a component of the PFR.²

Studies of the expression and copy numbers of the four par genes (Figs. 4 and 5) indicated that the levels of the four PAR proteins may be the result of homeostatic regulation of their respective RNA levels. The relative intensity of signals observed on Western blots of both purified PFR and lysates of epimastigotes probed with antibodies specific for the four PFR proteins (Figs. 1 and 7) suggest that PAR 1, PAR 2, and PAR 3 are present in approximately equimolar amounts, while PAR 4 is slightly less abundant. Maintenance of this stoichiometry appears not to be a function of gene frequency, since the re-

² A. K. Tiwari and J. E. Manning, unpublished data.
The view that the two PFR bands observed with PFR protein on SDS-PAGE contain only two major protein bands of apparent molecular masses 68 and 70 kDa is not evident. Nevertheless, in addition to sharing only two apparent molecular weights on SDS-PAGE, these four proteins also share only two predicted isoelectric points (Table I) and have very similar molecular weights on SDS-PAGE, these four proteins also share only two apparent molecular masses 68 and 70 kDa (4). It is our belief that the common physical and chemical properties shared by the four PAR proteins, along with the high aa similarity between PAR 2 and PAR 3, collectively have led to the generally accepted conclusion that the two major protein bands observed with PFR protein on SDS-PAGE contain only two different proteins (4, 6, 8–10). The view that the two PFR protein bands contain two distinct, but immunologically related proteins is based on several different findings. Monoclonal and polyclonal antibodies against these proteins have been found to react with both protein bands equally (8, 9), suggesting that the two proteins share common epitopes. Separation of the proteins in the two bands by use of standard biochemical procedures has been mostly unsuccessful, suggesting that the two proteins share common chemical properties (6, 7). Genes coding for two PFR proteins in T. brucei, PFR-A and PFR-C, have been cloned, and analysis of the protein sequences conceptually encoded by these genes reveals the two proteins to be highly similar in both their aa sequence and biochemical properties. Further, PFR-C has been shown to be present in the slower migrating PFR band on SDS-PAGE, while PFR-A is found in the faster migrating band. It follows that these two proteins likely would share common immunological epitopes, thus explaining the immunological cross-reactivity observed between the two protein bands. Although these studies clearly support the conclusion that two distinct but related proteins are present in the two PFR bands, they do not conflict with our finding of four distinct proteins, two of which, PAR 2 and PAR 3, share common epitopes (Table II). It also is likely that T. brucei may contain a protein similar to PAR 1, since PAR 1-specific monoclonal antibodies recognizes a protein of apparent mass 70 kDa in lysates of T. brucei (Fig. 8). Whether T. brucei also contains a protein similar to PAR 4 remains unknown, since the high specificity of monoclonal antibodies precludes interpreting the lack of reactivity of PAR 4-specific monoclonal antibodies with the T. brucei lysate as evidence that PAR 4 is not present in this parasite.

Analysis of the predicted aa sequence of the PAR proteins reveals that all share several common chemical and physical characteristics. The predicted molecular weights of the four proteins are similar (Table I), a fact that likely contributes to the observation that all four proteins migrate in SDS-PAGE as only two major protein bands of apparent molecular masses 68 and 70 kDa (4). However, since their migration pattern does not correspond directly with their predicted molecular weights, a definitive explanation for the observed separation of individual protein species into two distinct bands still is not evident. Nevertheless, in addition to sharing only two apparent molecular weights on SDS-PAGE, these four proteins also share only two predicted isoelectric points (Table I) and have very similar aa compositions (i.e. 39–41% charged aa, 14–17% basic aa, 22–26% polar aa, and 31–34% hydrophobic aa). Thus, it is not surprising that separation of these proteins from one another by use of standard biochemical techniques has been difficult.

Based upon a calculation of aa similarity, the dendrogram shown in Fig. 3 indicates a clear genetic lineage between PAR 2 and PAR 3. Also suggested is a distant relationship with PAR 1, while PAR 4 appears to be unrelated to the other PAR proteins. However, as discussed above, analysis of the aa composition of these proteins shows them to share common chemical properties. Possibly of equal importance, analysis of the predicted secondary structure of these proteins using five separate algorithms (23–27) indicates repeating coil/helix motifs throughout the entire length of all four proteins (unpublished). Thus, although considerable heterogeneity may exist between these proteins with respect to their primary aa sequence, they all appear to share common chemical and secondary structural features. Interestingly, the high helical content, repeating coil/helix motif, and primary sequence heterogeneity found in these proteins are also present in many filamentous proteins that interact to form high molecular weight heteropolymers (28). Thus, the similarity between the structural features of the PAR proteins and other filamentous proteins provide a compelling

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**TABLE III**

| Recombinant DNA clone | PAR gene | Origin of clone | Length of T. cruzi DNA insert |
|-----------------------|----------|----------------|------------------------------|
| λTecPar1a             | par 1    | Epimastigote cDNA library | bp                           |
| λTecPar1b             | par 1    | Epimastigote cDNA library | 1037                         |
| pTccPar1a             | par 1    | Epimastigote cDNA library | 2034                         |
| pTccPar1b             | par 1    | Epimastigote cDNA library | 2034                         |
| pTccPar1c             | par 1    | Epimastigote cDNA library | 2216*                        |
| pTccPar3a             | par 3    | Epimastigote cDNA library | 1980*                        |
| pTccPar3b             | par 3    | Epimastigote cDNA library | 1962*                        |
| pTccPar3c             | par 3    | Epimastigote cDNA library | 1976*                        |
| pTccPar4a             | par 4    | Epimastigote cDNA library | 1977*                        |
| pTccPar4b             | par 4    | Epimastigote cDNA library | 1980*                        |
| pTccPar4c             | par 4    | Epimastigote cDNA library | 2223                         |

* Denotes that the T. cruzi DNA insert encodes the entire conceptual PAR protein.
possibility that heteropolymers composed of these four proteins provide the basic building blocks for formation of the paraflagellar rod. With the availability of both the recombinant PAR proteins and their genes, the answer to this question as well as others related to the organization and assembly of the paraflagellar rod will be amenable to both in vivo and in vitro investigation.

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