Genetic dissection of the *Leishmania* paraflagellar rod, a unique flagellar cytoskeleton structure

**John A. Maga**, Trevor Sherwin, Susan Francis, Keith Gull and Jonathan H. LeBowitz.

1Department of Biochemistry, Purdue University, West Lafayette, IN 47907, USA
2School of Biological Sciences, University of Manchester, Manchester, UK

*Author for correspondence (e-mail: lebowitz@biochem.purdue.edu)

Accepted 8 June; published on WWW 21 July 1999

**SUMMARY**

The paraflagellar rod (PFR) is a unique network of cytoskeletal filaments that lies alongside the axoneme in the flagella of most trypanosomatids. While little is known about how two major *Leishmania mexicana* PFR protein components, PFR1 and PFR2, assemble into this complex structure, previous analysis of *PFR2* null mutants demonstrated that the PFR is essential for proper cell motility. The structural roles of PFR1 and PFR2 are now examined through comparison of *PFR2* null mutants with a new *PFR1* null mutant and *PFR1/PFR2* double null mutant parasites. Both PFR1 and PFR2 were essential for PFR formation and cell motility. When elimination of one PFR gene prevented assembly of a native PFR structure, the other PFR protein accumulated at the distal flagellar tip. Comparison of PFR substructures remaining in each mutant revealed that: (1) fibers that attach the PFR to the axoneme did not contain PFR1 or PFR2, and assemble in the absence of a PFR. (2) PFR1 was synthesized and transported to the flagella in the absence of PFR2, where it formed a stable association with the axoneme attachment fibers. (3) PFR2 was synthesized and transported to the flagella in the absence of PFR1, though it was not found associated with the axoneme attachment fibers. (4) PFR1 and PFR2 were located throughout the subdomains of the PFR. These data suggest that while PFR filaments contain both PFR1 and PFR2, the PFR is attached to the axoneme by interaction of PFR1 with the axoneme attachment fibers.

Key words: Paraflagellar rod, *Leishmania*, Flagellum, PFR1, PFR2

**INTRODUCTION**

Flagellar assembly is an intricate process that coordinates transportation and organization of protein components into a complex organelle. Creation of the canonical 9+2 axoneme structure is the central process of eukaryotic flagella biogenesis and has been extensively studied in model systems such as *Chlamydomonas* (Johnson and Rosenbaum, 1993; Dutcher, 1995). Some protozoan parasites, however, must also accommodate synthesis of a novel cytoskeletal structure called the paraflagellar rod (PFR) (Bastin et al., 1996b). The PFR is a massive lattice-like structure that runs adjacent to the axoneme, attached to axonemal microtubule doublets 4 through 7. Found in kinetoplastids, euglenoids, and some dinoflagellates, this unique and complex structure is critical for *Leishmania mexicana* and *Trypanosoma brucei* cell motility (Santrich et al., 1997; Bastin et al., 1998), though little is known about its molecular assembly or its role in the lifecycle of these important human pathogens.

Ultrastructural studies on trypanosomatid *Phytomonas* and *Herpetomonas* reveal three PFR regions: proximal, intermediate, and distal, relative to the axoneme (Farina et al., 1986). The proximal and distal regions share similar structures with each containing interdigitated 25 nm double filaments and a single thin 7 nm filament. The intermediate region contains thin filaments that connect the proximal and distal regions. Short attachment fibers shaped like the letters V or I connect the proximal region to the axoneme. This basic PFR structural organization has been corroborated in a study of *L. major*, though some differences in details of filament size, spacing, and placement were reported (al-Shammary et al., 1995).

The two major PFR proteins identified in *Crithidia fasiculata*, *T. brucei*, *T. cruzi*, *L. amazonensis*, *L. mexicana*, and *Euglena gracilis* migrate in SDS-PAGE as a doublet of equal staining bands with electrophoretic mobilities ranging from molecular masses 70,000-80,000 and 68,000-72,000 (Hyams, 1982; Russell et al., 1983; Ismach et al., 1989; Saborio et al., 1989; Beard et al., 1992; Deflorin et al., 1994; Fouts et al., 1998). Additional protein components of the PFR have also been identified (Woods et al., 1989; Woodward et al., 1994; Imboden et al., 1995; Fouts et al., 1998).

Genes encoding the major *L. mexicana* PFR protein components, PFR1 and PFR2, predict molecular masses of 74 kDa and 69 kDa, respectively and pl 5.3 (Moore et al., 1996; Pallinti, L. L. Moore and J. H. LeBowitz, unpublished). PFR1 and PFR2 protein sequences are about 60% identical, and each...
share over 80% amino-acid identity with their respective homologues from *T. brucei* (PFR-C, PFR-A) and *T. cruzi* (PAR 3, PAR 2). Computer algorithms predict a high alpha-helical content (>80%) and the potential for these helices to form coiled-coil motifs in all members of the PFR1/PFR2 family (Ngo and Bouck, 1998).

A functional role of the PFR is demonstrated in mutants lacking *L. mexicana* PFR2 (Santrich et al., 1997). Targeted PFR2 gene replacements produce parasites lacking an intact PFR, causing a large reduction in swimming velocity. Analysis of flagellar beat patterns in the PFR2 null mutant reveal severe perturbations in flagellar wave propagation that account for the motility defect and are consistent with the PFR providing internal elastic bending resistance to flagella (Santrich et al., 1997). *T. brucei* mutants lacking expression of the PFR2 homologue also display reduced cell motility (Bastin et al., 1998).

*L. mexicana* and *T. brucei* without PFR2 lack most of the PFR structure, though in each case a PFR substructure remains attached to the axoneme (Santrich et al., 1997; Bastin et al., 1998). This substructure resembles the proximal region of a normal PFR with respect to axoneme attachment, size, and general shape. These initial mutants show that it is possible to generate large structural defects in the PFR, and suggest that gene ablation would be a valid medium for studying PFR structure as well as the general principles of flagella morphogenesis.

This approach of removing protein components from a cytoskeletal structure has proven pivotal to the understanding of *Chlamydomonas* (Dutcher, 1995), and we have adopted it to understand PFR assembly in *Leishmania*. The present study compares PFR substructures in *Leishmania* double null mutants with newly constructed PFR1 null mutant and PFR1/PFR2 double null mutant parasites. Differences in mutant PFR substructure morphology suggested separate roles for PFR1 and PFR2 in PFR assembly, though immunolocalization of epitope-tagged PFR1 and PFR2 revealed a similar domain distribution of the two proteins.

**MATERIALS AND METHODS**

**DNA manipulation**

Gene targeting DNA fragments

Plasmid pR15′3′Flanks contained 3.6 kb of 5′-flanking and 2.1 kb of 3′-flanking sequence from the *PFR1* locus in vector pBluescript SKII(+) (Stratagene, La Jolla, CA, USA). 5′ flank DNA was the BamHI to EcoRI fragment from the pB6-1 SalI subclone of genomic DNA (V. Pallinti, L. L. Moore and J. H. LeBowitz, unpublished; Fig. 1A) with the BamHI site blunt with T4 DNA polymerase. 3′ flank DNA was the NsiI to EcoRI fragment from the pA9-1 MluI subclone treated with T4 DNA polymerase (Pallinti, L. L. Moore and J. H. LeBowitz, unpublished; Fig. 1A). The 5′ flank was ligated to Smal and EcoRI sites of pBluescript SKII(+). Then the 3′ flank was ligated into the HincII vector polylinker site to create pR15′3′Flanks. The polylinker EcoRV site between the two flanks in plR15′3′Flanks was used for ligation of drug resistance gene cassettes blunt with the large fragment of *E. coli* DNA polymerase I (Klenow) for creation of gene replacement targeting constructs. The NEO cassette was the 3.2 kb HindIII to BamHI fragment from pX63NEO (LeBowitz et al., 1991). The HYG cassette was the 2.1 kb HindIII to PpuMI fragment from pX63HYG (Cruz et al., 1991). The PHLEO cassette (containing the gene Sh-ble) was the 2.8 kb HindIII to BamHI fragment from pX63PHLEO (Freedman and Beverley, 1993). The SAT cassette was the 2.3 kb SalI to SpeI fragment from pLexSAT (Joshi et al., 1995). The resulting plasmids were named pR1KONEO, pR1KOHYG, pR1KOPHLEO, and pR1KOSAT. Digestion of each plasmid with XbaI and ApaI released the respective linear targeting fragment.

**Complementation vectors**

Plasmid pPFR1 was constructed as follows: PCR of a 760 bp *PFR1* fragment spanning the start codon and BgIII site of PFR1D (V. Pallinti, L. L. Moore and J. H. LeBowitz, unpublished; Fig. 1A) was performed to incorporate a Ndel site at the start codon and an EcoRI site upstream of the start codon. This fragment was ligated at the BgIII site to the 2.25 kb BgIII-EcoRV fragment containing the 3′ end of *PFR1D* and 1.2 kb of 3′ untranslated region from plasmid pA9-1 (V. Pallinti, L. L. Moore and J. H. LeBowitz, unpublished; Fig. 1A). This PFR1 cassette fragment digested with EcoRI and EcoRV was ligated into the *EcoRI* and Klenow-treated BamHI sites of pBluescript SKII(+).

Plasmid pPFR2 was constructed as follows: PCR of a 204 bp *PFR2* fragment spanning the start codon and PpuMI site of PFR2C (Moore et al., 1996) was performed to incorporate a Ndel site at the start codon and an EcoRI site upstream of the start codon. This fragment was ligated to the 1.67 kb PpuMI-BamHI fragment from p3.8kbClu (Moore et al., 1996) containing the 3′ end of PFR2C and downstream Mael site. This PFR2 cassette fragment was digested with EcoRI and BamHI and ligated into the same sites of pBluescript SKII(+).

Plasmid pPFR1PAC, used in complementation of line Δpfr1, contained the Klenow-treated 3 kb Ndel-XbaI fragment from pPFR1 ligated into the Klenow-treated BamHI site of pX63PAC.

Plasmid pPFR2PAC, used in complementation of line Δpfr2, was the Klenow-treated 3.5 kb ClaI fragment containing PFR2B and 5′ untranslated region from plasmid p3.5Clu (Moore et al., 1996) ligated into the Klenow-treated BamHI site of pX63PAC (Freedman and Beverley, 1993).

Plasmids pPFR2BB2PAC, used to introduce epitope-tagged pfr2 into line Δpfr2, was constructed as follows: the 1.8 kb EcoRI-BamHI fragment from pPFR2 was ligated into the respective sites of vector pUC18 (New England Biolabs) yielding plasmid pPFR2PUC. Unphosphorylated sense oligo 5′-GGCCGAGGTGACACAGAAC-CAGGACCCCGTGGACGC-3′ and anti-sense oligo 5′-GGCCCAG-GTCCAGGGGTCTGTTGTGCTGACACC-3′ were annealed and cloned into the PFR2EagI site of vector pPFR2PUC, yielding plasmid pPFR2BB2. This modification of *PFR2* inserted after amino acid Ala11 the sequence EVHTNQDPLDAI containing the 10 amino acid BB2 epitope tag (Bastin et al., 1996a) and two Ala residues. The Klenow-treated 1.8 kb EcoRI-BamHI fragment from pPFR2BB2 was ligated into the Klenow-treated BamHI site of pX63PAC, yielding plasmid pPFR2BB2PAC.

Plasmid pPFR1BB2PAC, used to introduce epitope-tagged pfr1 into line Δpfr1, was constructed as follows: unphosphorylated sense oligo 5′-GGAGGTGCCACAGCAACCGacctgc-3′ and anti-sense oligo 5′-GGCGGTACCGCCGTGCTGCTGACACC-3′ were annealed and cloned into the PFR1 site of vector pPFR1BB2. This modification of *PFR1* inserted after amino acid Ile251 the sequence EVHTNQDPLDAI containing the 10 amino acid BB2 epitope tag followed by Ala and Ile. The Klenow-treated 1.8 kb EcoRI-EagI fragment from pPFR1BB2 was ligated into the Klenow-treated BamHI site of pX63PAC, yielding plasmid pPFR1BB2PAC.

Plasmid pPFR2PFR1PAC, used in complementation of line Δpfr2Δpfr1, was made as follows: the Klenow-treated 1.8 kb EcoRI-EagI fragment from pPFR1 was ligated into the EcoRV site of vector pTX (Kelly et al., 1992), yielding plasmid pPFR1TEX. The T4 DNA polymerase-treated 3.0 kb SacI-EagI fragment from pPFR1TEX was ligated into the Klenow-treated BglII site of pPFR2PAC, yielding pPFR2PFR1PAC.
Growth and transformation of parasites
Promastigotes of *L. mexicana*, who strain MYNC/BZ62/M379, were cultured as described (Moore et al., 1996). Parasite electroporations with 1 µg of linear DNA (for gene replacements) or 10 µg of plasmid DNA (for episomal complementations), and selection of *Leishmania* clonal transfecants were performed as described (LeBowitz, 1994). Drug concentrations used in selective plates were 30 µg/ml G418 (GibcoBRL, Gaithersburg, MD, USA), 30 µg/ml hygromycin B (Sigma, St Louis, MO, USA), 20 µg/ml phleomycin (Sigma), 25 µg/ml nourseothricin (Udo Gräfe; Hans-Knöll-Institute für Naturstoff-Forschung e.V., Jena, Germany), and 11 µg/ml puromycin (Sigma). Drug concentrations for parasites in culture were 30 µg/ml G418, 30 µg/ml hygromycin B, 20 µg/ml phleomycin, 25 µg/ml nourseothricin, and 5.4 µg/ml puromycin.

Construction of Δpfr1 and Δpfr1,2 Leishmania
The PFR1 locus in diploid *L. mexicana* contains a tandem array of four copies of the PFR1 coding sequence, designated PFR1A, B, C, and D (V. Pallini, L. L. Moore and J. H. LeBowitz, unpublished; Fig. 1A). In order to delete the entire PFR1 array, unique DNA sequences flanking the locus were linked to one of four selectable markers: neomycin phosphotransferase (NEO), hygromycin B phosphotransferase (HYG), Shb-le (PHLEO), or streptothricin acetyltransferase (SAT), conferring resistance, respectively, to aminoglycoside G418, hygromycin B, phleomycin, and nourseothricin. The linearized NEO-containing targeting fragment was introduced into *L. mexicana* promastigotes to eliminate one chromosomal array of PFR1 and create a putative heterozygous line. A G418-resistant colony designated line 140.4 was selected as the recipient for the HYG targeting fragment. Following transformation, two colonies (lines 167.2 and 168.1) resistant to both G418 and hygromycin B were analyzed by Southern blots (Fig. 1C,D,E, lanes 4-5) to verify the genotype of Δpfr1 mutants (Table 1). Southern analysis of line 140-4 (Fig. 1C,D,E, lane 3) revealed that all copies of PFR1 were absent, suggesting that both chromosomal arrays of PFR1 in this line were eliminated in a single transformation as has been previously observed (Gueres-Filho and Beverley, 1996). Absence of PFR1 in line 140-4 was verified by western blot analysis (data not shown). While further analysis was performed on Δpfr1 lines derived from line 140.4, two additional Δpfr1 lines derived from an independent, heterozygous line designated 140.1 (Δpfr1-1:NEO/PFR1) were indistinguishable from 140.4-derived Δpfr1 lines in terms of swimming phenotype and immunofluorescence microscopy with mAb 2E10 (data not shown). All Δpfr1 lines displayed an increased lag time compared to wild-type cells when stationary phase promastigotes were diluted into fresh medium, although doubling times were the same (data not shown).

Construction of Δpfr1,2 was achieved by sequential transformation of linear PHLEO- and SAT-containing PFR1 targeting fragments into Δpfr2 promastigotes (Santrich et al., 1997; Table 1). Following PHLEO targeting fragment transformation, a phleomycin-resistant colony designated line 125.1 was selected as the recipient for the SAT-containing targeting fragment. Genotypes of two colonies resistant to both phleomycin and nourseothricin (lines 174.2 and 175.3, Table 1) were verified by Southern blot analysis (Fig. 1C,D,E, lanes 7-8).

Chromosomal separation and southern blot analysis
*Leishmania* chromosomes were isolated as described (Beverley, 1988) and separated by clamped homogeneous electric field (CHEF) electrophoresis as described (Santrich et al., 1997) except with a 4 minute switch time for 40 hours at 4.5 V cm⁻¹ and 11°C. Capillary transfer of DNA to Nytran membranes (Schleicher and Schuell, Keene, NH) and probing was as described (Sambrook et al., 1989). [γ-³²P]dCTP labeled probes were prepared by random priming of gel-purified DNA templates as described (Wahl et al., 1987). The PFR1 probe template was the 3 kb EcoRI-XbaI fragment from pPFR1. The PFR2 probe template was the 1.8 kb *NdeI-BamHI* fragment from pPF2R. The NEO probe template was the 0.9 bp *BamHI-HindIII* fragment from p7NEO (Santrich et al., 1997). The HYG probe template was the 0.8 fragment from pX63HYG. The PHLEO probe template was the 0.6 kb *HindIII-PstI* fragment from pX63PHLEO. The SAT probe template was the 2.2 kb *SalI-SphI* fragment from pLexSAT.

Western blot analysis
Parasites were detergent extracted at room temperature for two minutes in 1% Nonidet P-40 (Sigma) in PEM buffer (0.1 M Pipes, 2 mM EGTA, 1 mM MgSO₄, pH 6.9) with protease inhibitors (0.4 µg/µl EDTA, 5 µg/ml peptatin A, 0.1 µg/ml leupeptin, 0.5 µg/ml Pefabloc SC; Boehringer Mannheim, GmbH, Germany) and centrifuged one minute at 14,000 rpm in a microfuge to generate soluble supernatant and cytoskeleton pellet fractions. Whole cell, soluble, and cytoskeleton protein samples were prepared and separated with 8% SDS-PAGE as described (Sambrook et al., 1989). 5.6×10⁶ cell equivalents were loaded in each lane. Immunoblotting was performed with an ECL labeling kit (Amersham Life Sciences, Arlington Heights, IL). Monoclonal antibody (mAb) 2E10, which recognizes both PFR1 and PFR2 proteins, was a kind gift from Diane McMahon-Pratt (Ismach et al., 1989). mAb L13D6, which recognizes PFR1, was a kind gift from Linda Kohl (Kohl et al., 1999).

Two-dimensional PAGE
Two-dimensional PAGE was performed as described (Burland et al., 1983) except that gels were fixed for four hours at 800 V prior to second dimension separation and samples were protected during preparation with a protease inhibitor cocktail (0.4 µg/µl EDTA, 0.4 µg/µl EGTA, 0.2 µg/µl leupeptin, 1 mg/ml phosphatase A, 10 µg/ml leupeptin, 174 µg/ml PMSF, 0.1 µg/µl TPCK, 50 µg/ml TLCK, Sigma). Gels were loaded with 8×10⁶ cell equivalents and were silver-stained or immunoblotted as above.

Video microscopy
Videos of swimming parasites were filmed on a Nikon XFA microscope with a ×20 bright-field objective, a Hamamatsu C2400-07 Newvicon camera (700 lines resolution) with an Argus 20 image processor, and recorded on a Panasonic AG 7750 SVHS recorder. Recordings were displayed at a final 2.1×10³-fold magnification to measure forward swimming velocity. For each cell line, velocities were measured for 10 parasites swimming without tumbling for at least 1 second.

Immunofluorescence microscopy
Immunofluorescence microscopy on *Leishmania* cells and cytoskeletons was performed as described (Santrich et al., 1997). Whole cells were normally air-dried before fixation with the exception of Fig. 4B where parasites were settled on slides in a wet chamber for 3 minutes before fixation. Images were photographed on an Olympus BX60 fluorescent microscope with a ×100 objective and an Olympus PM-30 camera (Olympus America, Woodbury, NY). Images were digitized using a Nikon LS 1000 35 mm film scanner (Shinagawa-Ku, Tokyo) and Adobe Photoshop 5.0 software (Adobe Systems, San Jose, CA).

Electron microscopy
Thin section transmission electron microscopy (TEM) on whole cell sections was performed as described (Santrich et al., 1997). Cytoskeletons were prepared for TEM by centrifuging mid-log phase cells at 1,000 g, washing once in phosphate buffered saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.2), then resuspending in PEM buffer with 1% Nonidet P-40 and incubating for 3 minutes. Cytoskeletons were pelleted as above, washed once in PEM, then fixed, stained, dehydrated, embedded, and sectioned as described (Santrich et al., 1997), except prior to dehydration, cytoskeleton...
pellets were embedded in 1.5% agarose (Sigma Type VII) to aid pelleting.

Immuno-TEM was adapted from the method of van Tuinen and Riezman (1987). Cells were pelleted by centrifugation at 1,000 g and fixed for 2 hours at 4°C in 3% paraformaldehyde, 0.5% glutaraldehyde in PBS. Cells were then washed three times in PBS, incubated for 30 minutes at room temperature in 50 mM ammonium chloride, and washed once in PBS. Cells were dehydrated through an ethanol series, then the ethanol was replaced step-wise with Lowicryl HM20 resin (Electron Microscopy Sciences, Fort Washington, PA). Resin was polymerized at 4°C above a long wavelength (360 nm) UV light for 48 hours. Thin sections were mounted on Formvar and carbon-coated nickel or gold-gilded 100 mesh grids, and blocked by floating grids on TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.3% Tween-20). Grids were then floated on primary antibody in TBS-B buffer (20 mM Tris, 150 mM NaCl, 1% BSA) overnight at 4°C, followed by three washes in TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.3% Tween-20). Grids were blocked again in TBS-TB for 5 minutes, incubated on secondary antibody (anti-mouse IgG gold conjugate, 10 nm, Sigma) in TBS-TB for one hour at room temperature, washed three times in TBS-T and once in water, then air-dried. Grids were stained in 2% TBS-T and once in water, then air-dried. Grids were stained in 2% TBS-TB for one hour at room temperature, washed three times in TBS-TB for 20 minutes at room temperature. Grids were incubated on secondary antibody (anti-mouse IgG gold conjugate, 10 nm, Sigma) in TBS-TB for one hour at room temperature, washed three times in TBS-T and once in water, then air-dried. Grids were stained in 2% aqueous uranyl acetate, followed by lead citrate. Sections were observed on either a Phillips EM-200 or Phillips EM-400 electron microscope.

RESULTS

PFR1 and PFR2 are each essential for formation of the PFR

In order to understand the relative contribution of PFR1 and PFR2 to PFR organization, PFR1 null mutants and PFR1/PFR2 double null mutants were generated through sequential targeted gene replacements. The ultrastructures of these FPRs were compared to those in PFR2 null mutants (Santrich et al., 1997) and wild-type cells. A summary of genotypes and abbreviations of L. mexicana mutants created for this study is listed in Table 1. Southern blot analysis confirmed the genotypes of the PFR1 and PFR1/PFR2 null mutants (Fig. 1).

Western blots using mAb 2E10 (Fig. 2A) on wild-type L. mexicana detected two protein species migrating as a doublet with apparent molecular mass 85,000 and 79,000. In Δpfr1 cells, the upper band was absent. In Δpfr2 cells, the lower band was absent. In Δpfr1,2 cells, both bands were absent. Upon complementation with episomes expressing the deleted genes, the absent protein bands were restored (data not shown). In both Δpfr1 and Δpfr2 cells, lower levels of the remaining band were detected as compared with wild-type cells. Based on these data, the upper 85,000 molecular mass band must be PFR1 and the lower 79,000 molecular mass band must be PFR2. These values were slightly higher than the predicted molecular masses of 74 kDa and 69 kDa, respectively.

Cellular proteins from wild-type and null mutant lines were also separated in two dimensions according to their isoelectric point and size (Fig. 3). Clusters of PFR1 and PFR2 proteins were identified based on immunoblot analysis (Fig. 3C,D) and comparison of silver-stained gels (Fig. 3E). Western blots probed with mAb 2E10 detected two major protein species with molecular mass and pl corresponding to PFR1 and PFR2, and two less abundant protein species, one each of slightly higher acidity and molecular mass than the major PFR1 and PFR2 species (Fig. 3C). As expected, two-dimensional blots reacted with mAb L13D6, specific for PFR1 (data not shown), detected the major and minor protein species with molecular mass corresponding to PFR1 (Fig. 3D).

The major and minor PFR1 species observed in wild-type gels were absent from Δpfr1 and Δpfr1,2 samples but remained in Δpfr2 gels, confirming their identities (Fig. 3E). Similar analysis of PFR2 species was complicated by the presence of several overlapping protein spots. Still, the major PFR2 species was clearly absent from Δpfr2 and Δpfr1,2 gels, but not from Δpfr1 gels (Fig. 3E). A less abundant protein remained in the PFR2 area of the Δpfr2 and Δpfr1,2 gels (Fig. 3E); however, we could not determine whether or not this protein coincided with the minor species recognized by mAb 2E10. No other obvious differences outside the PFR cluster were observed in wild-type and mutant gels (data not shown).

Δpfr1, Δpfr2 and Δpfr1,2 parasites each lacked a PFR (see Fig. 5 below) and displayed a characteristic PFR-minus phenotype: 4-5-fold slower swimming velocity and an altered flagellar beat waveform indistinguishable from previously described Δpfr2 cells (Table 2; Santrich et al., 1997, waveform data not shown). A wild-type PFR and wild-type phenotype were each restored by introduction of episomes expressing the deleted PFR genes (Table 2; Fig. 4D, waveform analysis and TEM data not shown). Consistent with previous results (Santrich et al., 1997), this episomal gene expression resulted in a complementation of between 70% and 80% of cells examined by immunofluorescence microscopy (data not shown).

Table 1. Summary of null mutant and complemented Leishmania lines

<table>
<thead>
<tr>
<th>Null mutants</th>
<th>Strain numbers</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δpfr1</td>
<td>167.2, 168.1</td>
<td>Δpfr1::NEO/Δpfr1::HYG</td>
</tr>
<tr>
<td>Δpfr2</td>
<td>13.2</td>
<td>Δpfr2::NEO/Δpfr2::HYG</td>
</tr>
<tr>
<td>Δpfr1,2</td>
<td>174.2, 175.3</td>
<td>Δpfr1::PHLEO/Δpfr1::SAT Δpfr2::NEO/Δpfr2::HYG</td>
</tr>
<tr>
<td>N/A</td>
<td>140.4</td>
<td>Δpfr1::NEO/Δpfr1::NEO</td>
</tr>
<tr>
<td>N/A</td>
<td>125.1</td>
<td>PFR1/Δpfr1::PHLEO Δpfr2::NEO/Δpfr2::HYG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complemented mutants</th>
<th>Strain number</th>
<th>Episomal construct</th>
<th>Parent line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δpfr1+PFR1</td>
<td>183.3</td>
<td>pPFR1PAC</td>
<td>167.2</td>
</tr>
<tr>
<td>Δpfr1+pfr1/1T</td>
<td>314.1</td>
<td>pPFR1B2PAC</td>
<td>167.2</td>
</tr>
<tr>
<td>Δpfr2+PFR2</td>
<td>14.10</td>
<td>pPFR2PAC</td>
<td>13.2</td>
</tr>
<tr>
<td>Δpfr2+pfr2T</td>
<td>191.2</td>
<td>pPFR2B2PAC</td>
<td>13.2</td>
</tr>
<tr>
<td>Δpfr1,2+pfr1/1T</td>
<td>227.1</td>
<td>pPFR2PFR1PAC</td>
<td>174.2</td>
</tr>
</tbody>
</table>
Genetic dissection of the paraflagellar rod shown). This reconstitution demonstrated that PFR1 and PFR2 are essential and non-redundant constituents of the PFR, and that the observed phenotypes and structural morphologies were directly attributed to the absence of the respective PFR proteins.

**PFR1 and PFR2 are transported to the flagellar tip in the absence of each other**

When Δpfr2 cells were examined by immunofluorescence microscopy with mAb 2E10, a weak flagellar staining pattern, due to the presence of PFR1, was detected as compared with the intense flagellar staining observed in wild-type parasites (Fig. 4A; Santrich et al., 1997). Upon close examination of standard preparations of Δpfr2 cells, the faint PFR1 staining extended beyond the axoneme in linear or splayed fiber-like patterns (Fig. 4A). Under the same conditions, mutant as well as wild-type parasites observed by immunofluorescence microscopy with a mAb against membrane-bound lipophosphoglycans revealed extensions of the flagellar membrane with the same general topology as described above (data not shown). When Δpfr2 parasites were prepared without drying prior to fixation they also displayed a weak flagellar staining as compared to wild type (Fig. 4B); however, under these conditions the PFR1 protein accumulated at the flagellar tip in a ball-like staining pattern. A corresponding pronounced dilation of the flagellar tip was observed by phase contrast microscopy of Δpfr2 cells, but not observed in wild-type cells (Fig. 4B). Although these two cell preparations revealed differences in the disposition of PFR1 staining at the flagellar tip (Fig. 4A,B), both showed an accumulation of PFR1 at the distal tip of Δpfr2 flagella.

Similar results were obtained with immunofluorescence of Δpfr1 cells. Whole cells dried prior to fixation displayed faint PFR2 staining beyond the flagellar tip in linear and splayed configurations, though no staining was detected along the...
axoneme (Fig. 4A). Δpfr1 whole cells not dried before fixation displayed faint PFR2 staining along the axoneme, and like Δpfr2 cells, an intense ball-like staining at the distal tip of the flagellum corresponded to a dilation of the flagellar tip observed by phase contrast microscopy of Δpfr1 cells (Fig. 4B).

As expected, no signal was detected with mAb 2E10 in Δpfr1,2 parasites or cytoskeletons (Fig. 4A-C), though a slight flagellar tip dilation was detected by phase contrast microscopy in cell preparations not dried prior to fixation (Fig. 4B).

Detergent extraction of Δpfr2 cells eliminated the PFR1 signal at the flagellar tip, though as previously reported, the PFR1 signal along the axoneme remained indicating the presence of PFR1 in the cytoskeleton of these flagella (Fig. 4C; Santrich et al., 1997). In contrast, the PFR2 signal within the detergent extracted Δpfr1 cells was completely abolished (Fig. 4C). These results were supported by western blots of fractionated soluble and cytoskeleton protein (Fig. 2B). mAb 2E10 detected PFR1 in the cytoskeletal fraction of Δpfr2 cells, but PFR2 was detected only in the soluble fraction of Δpfr1 cells.

Together these data indicate that PFR1 and PFR2 are transported to the flagellum independently when the other protein is absent, and detergent soluble forms of these proteins accumulate at the distal tip of the flagellum.

Δpfr1,2 contains filaments attaching the PFR to the axoneme

PFR structures in wild-type, Δpfr1, Δpfr2 and Δpfr1,2 parasites were compared using thin-section transmission electron microscopy (TEM) (Figs 5, 7). Thin sections of whole Δpfr1,2 flagella revealed a small amount of material located alongside axonemal microtubule doublets 4 through 7 (Fig. 5, cells). Detergent extraction of cells prior to fixation removed much of this, leaving behind a distinct cytoskeletal substructure attached primarily to microtubule doublet 6 in more than 90% of examined sections, and less often to doublets 4, 5, and 7 (Fig. 5, cytoskeletons, Fig. 6). In cytoskeletal longitudinal thin sections, this Δpfr1,2 substructure bore a striking resemblance to the attachment fibers connecting the axoneme to a wild-type

Fig. 2. Western blot analysis. (A) Wild-type (WT), Δpfr1 (Δ1), Δpfr2 (Δ2), and Δpfr1,2 (Δ1,2) cellular protein samples were probed with mAb 2E10 which recognizes both PFR1 and PFR2. Marker positions (kDa) are left of the blots; PFR1 and PFR2 bands are indicated with arrows labeled 1 and 2, respectively. (B) Total cellular protein (T) was separated alongside cell equivalents of soluble (S), and cytoskeleton pellet (C) fractions and probed with mAb 2E10. Equal loading of protein samples in each lane was verified by silver staining duplicate gels (data not shown). Incubation of samples with detergent during fractionation increases proteolysis of soluble proteins. A band corresponding to a proteolytic fragment appears below the PFR2 band in the soluble fractions.

Fig. 3. Two-dimensional PAGE. Total protein samples were separated based on isoelectric point (pH horizontal axis) and size (SDS-PAGE vertical axis). (A) Complete silver stained gel of wild-type parasite protein. An area containing the PFR protein cluster is boxed. Molecular mass markers (Sigma) are labeled vertically (kDa) and pH is labeled horizontally. (B) Enlargement of PFR protein cluster from A. Western blots of the PFR cluster from wild-type cells are probed for PFR1 and PFR2 with mAb 2E10 (C), and for only PFR1 with mAb L13D6 (D). (E) Comparison of silver stained wild-type and null mutant PFR protein clusters. Major isoforms of PFR1 and PFR2 are labeled with arrows beside black numbers 1 and 2, respectively. Areas missing major isoforms of PFR1 and PFR2 are labeled with arrows beside gray numbers 1 and 2, respectively.
PFR proximal domain with respect to fiber length and spacing (Fig. 7). This suggested that the fibers connecting the PFR to the axoneme did not contain PFR1 and PFR2, and were assembled primarily to axonemal microtubule doublet 6 in the absence of a PFR structure.

The PFR substructures remaining in Δpfr1 flagella were indistinguishable from the Δpfr1,2 substructures when examined by thin-section TEM (Fig. 5). Consistent with the immunofluorescence microscopy and western blot data described above, this result indicated that PFR2 was not assembled into the flagellar cytoskeleton in the absence of PFR1.

The Δpfr2 PFR substructure was often similar to Δpfr1 and Δpfr1,2 substructures and attached primarily to axonemal microtubule doublet 6 (Fig. 6); however, Δpfr2 flagella were twice as likely to contain larger and more elaborate substructures than Δpfr1 and Δpfr1,2 when observed by thin-section TEM (Fig. 5) and negatively stained whole mount TEM (data not shown). As previous work demonstrates that PFR1 is present in this Δpfr2 PFR cytoskeleton substructure (Santrich et al., 1997), these data suggested that PFR1 was responsible for the larger Δpfr2 substructure, and that PFR1 formed a stable interaction with the axoneme attachment fibers found in the absence of PFR1 and PFR2.

**PFR1 and PFR2 are present throughout the PFR subdomains**

A model of PFR organization consistent with the above data is that PFR1 interacts with the axoneme attachment fibers because it is the primary structural protein of the proximal domain filaments, whereas PFR2 resides in the intermediate or distal domains. Elimination of PFR1 would theoretically disrupt proximal domain attachment to the axoneme and prevent assembly of PFR2 into a detergent insoluble complex. Alternatively, PFR1 and PFR2 could both be distributed throughout the PFR, with only PFR1 involved in axoneme attachment. Again, elimination of PFR1 would prevent assembly of PFR2 into a detergent resistant cytoskeletal structure.

These models could be distinguished by substructural localization of PFR1 and PFR2 within the PFR. To accomplish this, genes encoding each protein were modified to insert a 12 amino acid epitope (Bastin et al., 1996a) within the translated proteins. These epitope-tagged pfr1 and pfr2 constructs were used to complement their respective null mutant lines, creating lines Δpfr1+pfr1T and Δpfr2+pfr2T (Table 1). Each of these lines displayed swimming velocities similar to wild-type
parasites, demonstrating that the modified proteins were functional (Table 2). The complemented PFR structures were unaffected by expression of the epitope-tagged PFR proteins as verified by electron microscopy and immunofluorescence microscopy with mAb 2E10 (data not shown). Immunofluorescence microscopy on wild-type, Δpfr1+pfr1T and Δpfr2+pfr2T cells and cytoskeletons with an anti-epitope mAb (Bastin et al., 1996a) showed a strong flagellar signal in the complemented lines, and no detectable signal in wild-type parasites (Fig. 4C), demonstrating antibody specificity for the tagged proteins.

Immuno-electron microscopy using anti-epitope antibody on Δpfr1+pfr1T thin sections revealed the presence of tagged pfr1 throughout the proximal, intermediate, and distal domains of the PFR (Fig. 8A,B,C). Examination of sections of Δpfr2+pfr2T cells revealed a similar localization for tagged pfr2 (Fig. 8D,E,F). These data are consistent with the organizational model where PFR1 and PFR2 are both present throughout the PFR, though only PFR1 is involved in attachment to the axoneme.

![Fig. 5. TEM of whole cell (cells) and detergent extracted cytoskeletal (+NP40) thin cross-sections of flagella from wild-type and null mutant Leishmania. Axonemes are arranged with microtubule doublet 1 at the 12 o’clock position and subsequent doublets numbered counterclockwise. Bar, 100 nm (for all panels).](image)

![Fig. 6. Location of attachment fibers on null mutant axonemes. Sections of 150 flagella from Δpfr2 (black bars), Δpfr1 (gray), and Δpfr1,2 (white) cytoskeletons were observed by TEM and scored for the presence and location of PFR attachment fibers extending from the axoneme. The percentage of axonemal microtubule doublets containing an attachment fiber was recorded.](image)

![Fig. 7. Longitudinal flagellar sections of wild-type (A) and Δpfr1,2 (B) cytoskeletons. Bracket a, axoneme; f, attachment fibers; p, PFR. In this image, the wild-type section does not go through the center of the axoneme (the central microtubule doublet is not observed). This accounts for the narrow wild-type axoneme compared to the Δpfr1,2 axoneme. Bar, 100 nm (A and B).](image)
DISCUSSION

We have used genetic manipulation to selectively remove PFR1 and/or PFR2 from the PFR structure in *L. mexicana* and demonstrated that these proteins are essential and non-redundant constituents of the PFR. Examination of the resulting mutant PFR substructures has begun to yield clues to the organization of the native PFR structure.

Axoneme-PFR attachment fibers

The PFR in wild-type *Leishmania* promastigotes is attached to the axoneme by a series of regularly spaced connection fibers shaped like the letters I or V that join the proximal PFR domain to microtubule doublets 4-7. In Δpfr1,2 flagella, the PFR is completely ablated. Longitudinal and cross sections of these flagella revealed an axoneme with regularly spaced fibers extending primarily from microtubule doublet 6. These fibers resembled the axoneme connection filaments, though it remains unclear why this attachment is seen less frequently on mutant doublets 4, 5, and 7. Absence of PFR1 or PFR2 may leave attachments to doublets 4, 5, and 7 more susceptible to detergent solubilization, or lack of PFR1 or PFR2 in the assembly process may hinder attachment filament formation across all four microtubule doublets. Nevertheless, it is clear that these attachment fibers could be assembled in the absence of a PFR, and they contained neither PFR1 nor PFR2. The identities of the attachment fiber protein components are unknown; however, candidate proteins include homologues of *T. cruzi* PAR1 and PAR4 (Fouts et al., 1998), as well as *T. brucei* I17, which is reported to localize between the PFR and axoneme (Imboden et al., 1995).

PFR1 and PFR2 assembly

Several data indicated that PFR1 can attach to the axoneme connection fibers in the absence of PFR2: western blots showed PFR1 in the Δpfr2 cytoskeleton; immunofluorescence microscopy showed this cytoskeletal PFR1 localized to the Δpfr2 flagella; immuno-EM showed PFR1 in the Δpfr2 PFR subassembly (Santrich et al., 1997); and electron microscopy of whole cell and cytoskeleton sections showed the PFR substructure in Δpfr1 flagella larger compared to the Δpfr1,2 substructure.

Conversely, parallel experiments suggested that PFR2 does not attach to the attachment fibers in the absence of PFR1: western blots failed to detect PFR2 in the Δpfr1 cytoskeleton; immunofluorescence microscopy showed PFR2 was transported to Δpfr1 flagella, but PFR2 was not detected in cytoskeleton preparations; and electron microscopy of whole cells and cytoskeletons showed the PFR substructure in Δpfr1 flagella was indistinguishable from the Δpfr1,2 substructure.

The conclusion that PFR1, but not PFR2, interacts with axoneme attachment filaments suggested the possibility that these two proteins might be located within discrete PFR substructures. Immuno-electron microscopy, however, showed no discernable difference in the locations of PFR1 and PFR2. PFR1 and PFR2 could plausibly form heterodimer building blocks for filaments throughout the PFR, or be present as discrete filaments that are distributed throughout the PFR. In either case our results suggest that PFR1 mediates contact with the axoneme attachment fibers. Future biochemical or yeast two-hybrid experiments with attachment filament components,

Fig. 8. PFR 1 and PFR2 substructural localization. Immuno-TEM of thin sections show labeling of epitope-tagged pfr1 in line Δpfr1+pfr1T (cross sections, A,B; longitudinal section, C) and of epitope-tagged pfr2 in line Δpfr2+pfr2T (cross sections, D,E; longitudinal section, F). Bars, 100 nm.
PFR1 and PFR2 should aid in detailing these protein-protein interactions.

**PFR1 and PFR2 targeting and transport**

Addition of new axoneme components in *Chlamydomonas* occurs at the distal end of flagella (Johnson and Rosenbaum, 1992), requiring transportation of proteins along the flagellum to the site of assembly. In this process of intraflagellar transport, rafts of protein components travel between the axoneme and the flagellar membrane and are dependent on the activity of motor proteins (Rosenbaum et al., 1999). While observing dividing *Leishmania*, we found that the PFR of growing daughter flagella was always similar in length to the adjacent axoneme, suggesting that the two structures were assembled concurrently (unpublished results). It is therefore expected that the protein constituents of the PFR require transport to the distal tip of growing flagella.

In *Apfr1* and *Apfr2* cells, remaining PFR2 or PFR1 protein accumulated at the distal end of the flagella. Presumably, these remaining PFR proteins are correctly transported to the distal end of the flagellum, but accumulate there in the absence of PFR assembly. A similar phenomenon is observed in a *T. brucei* PFR2 homologue mutant that displays accumulation of PFR protein in a similar dilated structure at the flagellar tip (Bastin et al., 1998). The presence of a slightly dilated flagellar tip in *Apfr1,2* parasites suggested that PFR components other than PFR1 and PFR2 might also accumulate at the flagellar tip in the absence of a PFR structure. Additionally, while some axonemal structures appear to be preassembled in the cytoplasm prior to flagellar localization (Rosenbaum et al., 1999), the presence of PFR2 and PFR1 in the flagella of *Apfr1* and *Apfr2* cells indicated that each protein contained information for independent flagellar localization.

Although some PFR1 and PFR2 protein accumulated in the flagellum in the absence of the other protein, the bulk of these proteins may be rapidly degraded. Western blots showed lowered levels of remaining PFR1 and PFR2 in the respective null mutants compared to wild-type protein levels. As we observed mRNA levels of PFR1 and PFR2 were unchanged in the null mutants (L. L. Moore, K. Mishra and J. H. LeBowitz, unpublished results), this suggested that the rate of PFR1 and PFR2 degradation was greater when these proteins are not incorporated into a native PFR.

Two-dimensional PAGE revealed one major isoform and at most only one minor isoform for both PFR1 and PFR2. While there may likely be additional PFR components in the *Leishmania* PFR cluster, this appeared to be less complex than the 10 flagellar rod isoforms identified in *Euglena* (Ngo and Bouck, 1998). The nature of the PFR isoforms and their biological relevance remain to be determined.

Identification of remaining PFR components is required for a more complete model of PFR organization. Given the relative ease of null mutant generation and the ability to reconstitute the PFR with genetically modified components, this *Leishmania* system provides an effective in vivo system for study of specific molecular details of PFR construction and function. The *Leishmania* Genome Project further promises to make available *Leishmania* axoneme components, allowing examination of axoneme organization and PFR-axoneme interactions in a genetically tractable organism.

We thank Diane McMahon-Pratt (Yale university) for her generous gift of mAb 2E10; Debra Sherman and Douglas Murphy (Purdue University) for helpful assistance and advice on electron microscopy and video microscopy; Charles Brokaw (California Institute of Technology) for darkfield microscopy analysis; Andrea Baines and Christiane Hertz-Fowler (University of Manchester) for assistance with 2D-PAGE; and Philippe Bastin (University of Manchester), David Asai (Purdue University), and Joann Otto (Purdue University) for helpful suggestions. J.H.L. is a Burroughs-Wellcome New Investigator in Molecular Parasitology. This work was supported by National Science Foundation grants 9724105-MCB and 9724752-INT to J.H.L. and by Wellcome Trust grants to K.G. This is paper #16007 from the Purdue Agriculture Research Station.

**REFERENCES**


Deforlin, J., Rudolf, M. and Seebeck, T. (1994). The major components of the paraflagellar rod of *Trypanosoma brucei* are two similar, but distinct proteins which are encoded by two different gene loci. *J. Biol. Chem.* 269, 28745-28751.


