J Parasit Dis (Oct-Dec 2015) 39(4):589–595
DOI 10.1007/s12639-014-0422-x

REVIEW ARTICLE

An overview on kinetoplastid paraflagellar rod

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Received: 6 September 2013 / Accepted: 13 January 2014 / Published online: 7 February 2014

Abstract Kinetoplastids, the evolutionary ancient organisms exhibit a rich and diverse biology which epitomizes many of the fascinating topics of recent interest and study. These organisms possess a multifunctional organelle, the flagellum containing a canonical 9 + 2 axoneme which is involved in vital roles, viz. parasite cell division, morphogenesis, motility and immune evasion. Since Antony Van Leeuwenhoek's innovative explanation of 'little legs' helping the movements of microbes in 1975, this biological nanomachine has captured the thoughts of scientists. The core structure of kinetoplastid flagellum is embroidered with a range of extra-axonemal structures such as paraflagellar rod (PFR), a large lattice like structure which extends alongside the axoneme from the flagellar pocket to the flagellar tip. The coding sequences for significant components of PFR are highly conserved throughout the Kinetoplastida and Euglenida. The high order organization and restricted evolutionary distribution of the PFR components and structure makes the PFR a particularly valuable therapeutic and prophylactic target. This review focuses on the recent developments in identification of ultra structural components of PFR in order to understand the function of this intriguing organelle and devising strategies for therapeutic interventions.

Keywords Flagellum · Kinetoplastida · Paraflagellar rod · Trypanosomes · Vaccine

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Introduction

The kinetoplastids among protists have received much attention from scientific community owing to their wealth of interesting cellular processes and zoonotic importance. Family trypanosomatidae (Order: Kinetoplastida) is composed exclusively of parasitic protozoa and includes the digenetic genera mainly *Leishmania* and *Trypanosoma* which are the causative agents of widespread diseases of both human and animals. These flagellate parasites are generally transmitted by hematophagus insects and have evolved to live within two challenging environments such as mammalian host and the insect digestive system. So far as *Trypanosoma* spp. is concerned, owing to its mechanical transmission by blood sucking flies, they have the widest geographical distribution infecting a wide range of hosts. In the Indian subcontinent the infection is prevalent in a wide range of wild and domestic animals including companions. Considerable variation in degree of endemicity is related to prevalence of fly vector, size of susceptible host population, prevalent agroclimatic conditions as well as the sensitivity of the particular diagnostic test applied (Singh and Tewari 2012; Pathak and Chhabra 2010; Singh and Chhabra 2008). The outcome of the infection varies with the host species infected, acute fever, anaemia, weight loss, reproductive disorders, immunological anergy, lymphadenopathy or death (Bajyana Songa et al. 1987; Rottcher et al.

1987; Taylor 1998; Holland et al. 2003; Tewari et al. 2009; Kurup and Tewari 2012) are the common features of infection. In this review, we will largely focus on studies involving trypanosomes as they assume a greater importance to biologists in terms of their unique ability of evading the host immune response by antigenic variation (Borst and Rudenko 1994; Singh et al. 1995; Donelson 2003) imposing a formidable challenge towards development of a protective vaccine against the disease. Since its discovery in 1880, though a plethora of publication has enriched the knowledge on different facets of its biology as well as molecular basis of antigenic variation, devising a foolproof strategy for blocking the sequential expression of variable antigenic surface epitopes in vivo has not yet been successful and therefore, vaccine development against animal trypanosomiasis based on variant surface glycoprotein no longer holds any promise (Donelson et al. 1998). Development of strategies for identification and application of invariant antigens as futuristic vaccine targets have been in the centre stage of trypanosome research which envisage a better way of management of the disease by effectively negotiating the pathological molecules of trypanosome origin. To materialize a fruitful search of a potentially protective vaccine target, it has now been increasingly essential to characterize the alternate target molecules more stringently and in reality the quest is going unabated world over. One such target is the paraflagellar rod (PFR) proteins present in the kinetoplastid flagellum. It is a complex network of cytoskeletal filaments extending alongside the axoneme from the flagellar pocket to flagellar tip. The significant components of PFR are highly conserved throughout the Kinetoplastida and Euglenida (Portman and Gull 2010). As chemotherapy and vaccine have enjoyed modest success in curbing their associated diseases, research into such unique targets of kinetoplastids is gaining greater interest from this perspective.

What is paraflagellar rod?

The flagella of all kinetoplastids contain a major accessory structure the PFR also known as the paraxial rod has been served as a focus since it was first identified (Vickerman 1962). This is a complex and highly organised lattice like cytoskeletal filaments that runs alongside the common (9 + 2) microtubular axoneme throughout its length except in the region within the flagellar pocket. Unlike the typical axoneme which is broadly conserved among eukaryotes, the PFR has only been observed in kinetoplastids, dinoflagellates and euglenoids (Cachon et al. 1988; Bastin et al. 1996, 2000; Maga and LeBowitz 1999). This structure is reduced in some symbiont- carrying trypanosomatids such as *Crithidia deanei* (Gadelha et al. 2005). PFR is present in all lifecycle stages of kinetoplastids with notable exception in the amastigote stages of *Trypanosoma cruzi* and *Leishmania* spp. where the remnant flagellum, with a very restricted axoneme, is confined to the flagellar pocket. Apart from its core structural components, PFR1 and PFR2 (Schlaeppi et al. 1989; Deflorin et al. 1994) and a few recently identified minor proteins, its exact function and basic molecular composition remains yet to be determined. But its high order organisation and restricted evolutionary presence justify this structure for a very specific function in these organisms.

Ultrastructure

Although the defining components of PFR appear to be conserved throughout Kinetoplastida and Euglenoid, its ultrastructure is variable in size between species and in some cases a significantly reduced PFR is present. Based on ultrastructural studies in related trypanosomatids, *Herpetomonas* and *Phytomonas*, it has been proved that PFR is a complex, trilaminar lattice like structure with three distinct zones like proximal, intermediate and distal, relative to the axoneme (Fuge 1969). Transmission electron microscopy reveals the proximal region as a simple structure while the intermediate and distal regions show precise orientations of thin and thick filaments whose arrangement is often characteristic of the species (De Souza and Souto-Padron 1980; Farina et al. 1986; Sant'Anna et al. 2005).The proximal and distal regions have the same general structure, being composed of dense plates of thin filaments of 7–10 nm and thick double filaments of 25 nm that intersect each other at an angle of 100°. The proximal and distal plates are connected by regularly spaced thin filaments which form the less electron dense intermediate zone. The intermediate region contains thin (5 nm) filaments that connect the proximal and distal regions intersect at an angle of 45°. The proximal domain of PFR is linked to the axonemal microtubule doublets 4–7 by electron dense filaments (Farina et al. 1986). These connections are seen as regularly spaced fibres of 40–50 nm long in V or Y shape in longitudinal sections. The overall diameter of the PFR is 150 nm throughout much of its length. The PFR is always positioned between the axoneme and the flagellar attachment zone (FAZ). The PFR and axoneme maintain a defined orientation in respect to each other with the central pair of microtubules having a steady position (Gadelha et al. 2006). The position of flagellum relative to the cell body is well defined with the PFR always seen in closer propinquity to the cell body. The association between PFR and axoneme is extremely strong and is not disrupted by non-ionic detergent, high salt treatment or hypotonic shock, but it is highly sensitive to trypsin treatment (De

Souza 1984). The growth of the PFR in the new flagellum is one of the most exciting features of the trypanosome cell cycle. It is precisely timed and correlated to the replication of DNA in the nucleus and the kinetoplast (Bastin and Gull 1999; Sherwin and Gull 1989). A PFR must be assembled in concert with the axoneme in the daughter flagellum with each cell division and developmentally regulated. The new flagellum, containing PFR, always originates from the posterior end of the trypanosome cell where as the old flagellum remains at the anterior most position. A detailed cell cycle analysis showed that the growth of the new PFR is first observed at 0.52 of the cell cycle in procyclic *Trypanosoma brucei* cells (Sherwin and Gull 1989). Further the assembly of PFR is developmentally regulated in some trypanosomatids like *Leishmania*. Insect stages of *Leishmania* possess a full length flagellum with PFR, whilst that of mammalian stage contains only an attenuated, non emergent flagellum completely lacking a PFR (Moore et al. 1996).

Composition

The unique structure and composition of the PFR, together with its role in cell motility, viability and immunology, made it an exciting area of research for therapy. Although major structural components of PFR have been described in several species of Kinetoplastida, the complete composition of this structure is still unknown. The ultra structure complexity of the PFR suggests a complex biochemical composition which was first revealed in *Crithidia fasciculata* and presented two major proteins PFR1 and PFR2 (Russell et al. 1983) and these two proteins were later observed by SDS-PAGE separation of purified flagella of *Herpetomonas megaseliae* (Cunha et al. 1984). The slow migrating protein band in SDS-PAGE gel was defined as PFR1 while the fast migrating band was called PFR2. Depending on the organism, the mobility (Mr) for PFR1 ranges from 70,000 to 80,000 Da and for PFR2 from 68,000 to 72,000 Da. The PFR1 and PFR2 genes from *T. brucei*, *T. cruzi* and *Leishmania mexicana* are highly conserved across species (over 80 % amino acid identity). Further it is interesting to note that both PFR1 and PFR2 proteins are components of native PFR antigen and do not share common B cell epitopes (Abdille et al. 2008). Taking into consideration of the high sequence homology between the major components of PFR among trypanosomatids (Maga and LeBowitz 1999), we assumed that the PFR gene could be highly conserved among *Trypanosoma* species and could be used as a common vaccine candidate. Keeping this in mind, we investigated the existence of PFR gene in *T. evansi*. Molecular cloning in our laboratory and subsequent nucleotide sequencing of PFR1 of *T. evansi* confirmed a high level of sequence homology of 99.8 % between the Izatnagar (India) and China isolates with change of only one nucleotide at 867 bp of PFR1 ORF further establishing its highly conserved nature. The nucleotide sequence homology was further assessed between the related species and genus revealing 99.8, 82.1, 79.9, 72.9 % homology with *T. brucei*, *T. cruzi*, *Leishmania infantum and Crithidia deanei*, respectively (Maharana et al. 2011a; Maharana and Tewari 2013). The deduced amino acid sequence of *T. evansi* PFR1 revealed 99.7 % homology between Indian and China isolate. It also showed 99.8, 92.7, 84.7, 82.4 % homology with *T. brucei*, *T. cruzi*, *L. infantum* and *C. deanei*, respectively (Maharana et al. 2011a). Subsequently, cloning of the entire ORF of PFR2 gene, using pDRIVE vector revealed the nucleotide sequence homology of 99.9 % between the Izatnagar and China isolates of *T. evansi*. Change of a single nucleotide was located at position 928 of PFR2 ORF in the Indian isolate (Maharana et al. 2011b, 2013). The nucleotide sequence also showed 99.9, 82.4, 75.3 and 74.8 % sequence homology with the published sequence of *T. brucei*, *T. cruzi*, *L. infantum* and *C. fasciculata*, respectively (Maharana et al. 2011b). The intron-less nature of both the genes was confirmed by amplification of the target sequence coding for PFR1 and PFR2 from the genomic DNA template of an equine isolate of *T. evansi* (Maharana and Tewari 2013). Sequence conservation is maintained throughout PFR1 and PFR2 with the exception of 20–30 residues of the N- and C-terminal sequences. A number of possible minor protein (more than 40) constituents with higher molecular weight (Mr 180,000 to >300,000 Da) that immunolocalize to the PFR have been described through biochemical, immunological and bioinformatics techniques. The nature of these components provides increasing evidence for a PFR role in regulatory, signaling and metabolic functions (Portman and Gull 2010). The genome sequencing projects of *T. cruzi*, *T. brucei*, and *L. major* have shown that the gene encoding PFR1 and PFR2 are distinct but related and are present in separate tandem arrays (Berriman et al. 2005; El-Sayed et al. 2005; Ivens et al. 2005) which are now considered as the defined core components of PFR. Sequence analysis reaffirms that the major PFR components are conserved in kinetoplastids and form a doublet of homologous proteins in most trypanosomatids which may be as a result of a single gene duplication event that predates the divergence of Kinetoplastida and Euglenida. Two major proteins, termed PFRA and PFRC in *T. brucei* have been purified from the PFR of trypanosomes, and the corresponding genes have been identified (Schlaeppi et al. 1989; Deflorin et al. 1994). Thereafter, orthologues have only been found in related kinetoplastids (*Trypanosoma*, *Leishmania*) (Beard et al. 1992; Moore et al. 1996; Fouts et al. 1998; Maga et al.

1999). Fouts et al. (1998) reported that the major structural proteins present in PFR of *T. cruzi* composed of four proteins, designated PAR 1, PAR 2, PAR 3 and PAR 4 which provide the basic building blocks for formation of PFR. They (proteins) like in other kinetoplastids migrate on SDS-PAGE gels as two separate electrophoretic bands. Further investigation using monoclonal and polyclonal antibodies against the four proteins encoded by these genes shows that PAR1 and PAR3 are present in slower migrating electrophoretic band and that of PAR2 and PAR4 only in faster migrating band. Analysis of nucleotide sequence and deduced amino acid sequence of these genes specify that PAR2 shares high sequence similarity with PAR3 and may be the members of a common gene family. Gadelha et al. (2004) introduced a reliable standard nomenclature for the major PFR genes and proteins in order to stay away from puzzling or ambiguous explanation. According to the consolidated nomenclature, the PFR1 orthologues are designated as PFR1 in *C. fasciculata, T. evansi*, *L. mexicana* and *L. major*, PFRC in *T. brucei* and PAR3 in *T. cruzi*, respectively. Similarly the PFR2 orthologues of the above species are designated as PFR2, PFRA and PFR2 respectively. The *T. cruzi* PAR2, *T. brucei* PFR A and *L. mexicana* PFR2 genes encode homologues whose predicted aminoacid sequences are identical between 80 and 90 %. The PFR C in *T. brucei* and PFR1 in *L. mexicana* show 77 % homology encoding the larger portion of PFR (Santrich et al. 1997). These reports from biological research further affirm the notion that the PFR is highly conserved among the kinetoplastids studied so far.

Functions

The function of PFR had been the subject of many reviews. PFR acts as a physical support to the flagellum i.e. as a thickening and stiffening agent (Fuge 1969). It also helps in attachment of kinetoplastid parasites to the insect host at some stages in their life cycle which is achieved via electron dense hemi-desmosome- like plaques associated with proximal portion of flagellum (Bastin et al. 1996; Maga and LeBowitz 1999). During the formation of attachment plaques, the anterior tip of the flagellum enlarges that contains the PFR and additional filaments that emerge from the main PFR. This morphological manipulations further strengthen a possible role of PFR during the crucial process of tissue attachment. The active role of PFR in the motility of the organism has been established by a distinct reduction in cell motility in the PFR ablated trypanosomatids or mutants without a native PFR structure and it has been demonstrated that PFR2 RNAi knock down mutants in procyclic *T. brucei* (Snl1) showed a dramatic decrease in flagellar wave frequency and amplitude that leads to a loss of cell motility and sediments at the bottom of culture flasks (Bastin et al. 1998). The PFR2 null mutant of *Leishmania* shows severe flagellar waveform perturbations, a shorter wavelength, including a decrease in frequency and reduced amplitude compared with the wild-type beat patterns (Santrich et al. 1997). Both sets of experiments indicate at a critical role of an intact PFR to flagellar and hence cellular motility. A high internal resistance is needed for propulsion of the parasite in an environment of high external resistance (i.e. high viscosity) like blood and lymph. Kinetoplastid encounter such environments during migration in the host bloodstream and the insect vector. The PFR provides energy for propulsion in such viscous medium owing to apparent association of an ATPase activity within it (Piccini et al. 1975). PFR helps in phosphotransfer relay to maintain the supply of ATP to the distal part of the flagellum further support the hypothesis of possible role of PFR in motility (Oberholzer et al. 2007; Ginger et al. 2008).The discovery of two PFR specific adenylate kinases support this above hypothesis (Pullen et al. 2004). The role of PFR as a regulatory and metabolic platform for control of both motile and sensory flagellar functions was supported after discovery of many enzymatic and Ca^{2+} regulated components (Portman et al. 2009). Apart from homeostatic and metabolic roles the PFR plays a crucial role in environment sensing and cell signaling in a variety of organisms. When the PFR2 is not correctly assembled, bloodstream form cells progress through multiple rounds of organellar replication but are unable to complete cytokinesis (Broadhead et al. 2006). The PFR is a feature of all cycle stages with the exception of the amastigote form of *T. cruzi* and *Leishmania* spp. where the remnant flagellum does not emerge from the flagellar pocket and does not present a PFR (Portman and Gull 2010). The fact that few kinetoplastids and certain monogenetic parasites lacking a PFR have regular flagellar beat patterns and are motile is enigmatic. Perhaps these organisms have developed a compensatory mechanism to provide internal flagellar resistance, or their habitat does not demand such a mechanism. Of course, other functions of the PFR remain to be discovered.

Role as a vaccine target

The diseases caused by trypanosomatids like human sleeping sickness and animal trypanosomiasis causes a huge economic loss to the livestock industry (Reid 2002). Control of the disease through preventive measures and improved management practices remains a mainstay as chemotherapy has shown only modest success due to emergence of parasitic drug resistance. There is mere hope of development of a new drug in the near future. Variation of the glycoproteins

between and within *Trypanosoma* species remains the main constraint in the vaccine development. So there is an urgent need to identify new effective drug and vaccine target. This has prompted researchers to find out invariant trypanosome components like PFR proteins universally present in the kinetoplastid flagellum as a potential drug and vaccine target (Taylor 1998; Abdille et al. 2008). The restricted evolutionary distribution of the PFR structure and components compared to the more conserved structure and components of the axoneme makes the PFR a particularly valuable target possibility for therapeutic intervention (Reviewed by Portman and Gull 2010) against parasites such as *T. cruzi* (causing Chagas disease), African trypanosomes (responsible for nagana and sleeping sickness) and *Leishmania* spp. (causing kala-azar or visceral leishmaniosis) of zoonotic importance (Hunger-Glaser and Seebeck 1997; Wrightsman et al. 1995). PFR components are highly immunogenic (Woods et al. 1989; Woodward et al. 1994; Kohl et al. 1999) and anti-PFR antibodies have been identified in infected animals (Imboden et al. 1995). More over, it is interesting to note that they bear no homology to any human and livestock animal proteins (Clark et al. 2005). PFR proteins have been demonstrated to be immunogenic when PFR2 was used alone (Saravia et al. 2004) and/or co-administered together with PFR1 and PFR2 against *T. cruzi* infection in mice (Luhrs et al. 2003). Immunisation with purified PFR proteins from *T. cruzi* epimastigote was shown to completely protect the mice from a subsequent challenge with fatal dose of this parasite (Wrightsman et al. 1995). However, the protection was dependent on the route of PFR vaccine delivery since inoculation through intra peritoneal route completely failed to protect, while inoculation the sub cutaneous route conferred immunity. Since antibodies from human patients suffering from Chagas's disease also recognised PFR proteins (Wrightsman et al. 1995; Michailowsky et al. 2003). Saravia et al. (2005) identified PFR2 as a potential vaccine target against *Leishmania* infection. Wrightsman and Manning (2000) reported that PFR antigen co-absorbed onto alum with rIL-12 or adenovirus expressed IL-12 could elicit a cell mediated immune response of Th1 type that provides protection against lethal challenge when subcutaneously delivered.

To answer the pertinent question of immune recognition of PFR, since it is a hidden antigen, it has been theorised that the degradation of flagellum and PFR during the transition from promastigote to amastigote form, the components are made available for immune recognition (Michailowsky et al. 2003; Saravia et al. 2005). Another possibility of exposure of the hidden antigens to the host immune system exists following death and lysis of the parasite. It has been hypothesized that specific antibodies against hidden antigens like PFR make their access through the flagellar pocket region.

Future perspectives

The PFR is a multi-functional organelle being involved in motility, morphogenesis, cytokinesis and parasite attachment to host tissues. It is an impressive target for therapeutic intervention of pathogenic trypanosomatids without any deleterious effect on their mammalian hosts. Our findings on sequence homology of nucleotides only further reaffirm the notion that vaccination with PFR molecule(s) could be effective not only in different strains within a trypanosome species but also against other species of the same genus. The immunogenic and protective effects of PFR protein of kinetoplastids need to be further explored in laboratory and other experimental animal models.

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