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Composition and sensory function of the trypanosome flagellar membrane

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Summary

A cilium is an extension of the cell that contains an axonemal complex of microtubules and associated proteins bounded by a membrane which is contiguous with the cell body membrane. Cilia may be nonmotile or motile, the latter having additional specific roles in cell or fluid movement. The term flagellum refers to the motile cilium of free-living single cells (*e.g.*, bacteria, archaea, spermatozoa and protozoa). In eukaryotes, both nonmotile and motile cilia possess sensory functions. The ciliary interior (cilioplasm) is separated from the cytoplasm by a selective barrier that prevents passive diffusion of molecules between the two domains. The sensory functions of cilia reside largely in the membrane and signals generated in the cilium are transduced into a variety of cellular responses. In this review we discuss the structure and biogenesis of the cilium, with special attention to the trypanosome flagellar membrane, its lipid and protein composition and its proposed roles in sensing and signaling.

Introduction

Most eukaryotic cells possess one or more unique organelles known as cilia, membranated projections that regulate cell motility and environmental sensing, and serve important roles in human health and disease [1]. Cilia are commonly classified as nonmotile (primary) or motile. All cilia share many structural and functional properties. An expanding group of human disorders, collectively known as the ciliopathies, has been linked to defects in ciliary structure, function and associated signaling pathways, generating renewed interest in the biology of these organelles [2]. The ciliopathies are widely diverse, ranging from embryonic patterning defects to renal and ocular diseases [3]. The subset of motile cilia includes flagella that primarily function in the propulsion of the single celled organisms, including trypanosomes (*Trypanosoma cruzi* and *brucei*) and *Leishmania*, flagellated, parasitic protozoa that cause a range of debilitating human diseases. The focus of this review is on common principles of ciliary structure, with special attention to the trypanosome flagellar membrane, its lipid and protein composition, and its proposed roles in sensing and signaling.

Ciliary structure

Eukaryotic cilia contain three distinct domains: the cytoskeleton axoneme, the soluble ciliary compartment (cilioplasm), and the ciliary membrane [4]. The central axoneme

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consists of a microtubular array that provides the structural support and gives the cilium its recognizable form. The most common axonemal arrangements includes the 9 + 2 arrangement, in which a central pair of microtubules is surrounded by nine outer double microtubules, and the 9 + 0 arrangement, in which the central pair of microtubules is absent. Positioned at the base of the axoneme is a barrel-shaped structure called the basal body that consists of nine triplet microtubules and serves as a differentiated centriole [5]. Transition fibers, sheet-like projections radiate outwards from the mature basal body and serve as a permeability barrier that restricts the free diffusion of vesicles and macromolecules between the cell cytoplasm and the soluble ciliary compartment [6]. This property of transition fibers enables the cilium to maintain its unique composition of lipids and proteins that is distinct from the cytoplasm, thereby leading to a vision of the cilium as a separate cellular organelle. The ciliary membrane wraps around the ciliary axoneme and conforms to its shape. It is a highly specialized domain of the plasma membrane which, while contiguous with cell body and ciliary pocket membranes, nevertheless maintains a unique lipid and protein composition and distinct functions [7].

Lipid composition of trypanosome flagellar membrane

The flagella of several microorganisms have been isolated to study their lipid composition in comparison to the cell body. These studies demonstrate that the flagellar membrane is enriched in sterols [8–10], glycolipids [11] and sphingolipids [12,13], all which are components of canonical lipid raft microdomains. Numerous lipid raft associated proteins from the kinetoplastids are also dually acylated. These include *T. brucei* calflagins (Tb17, Tb24 and Tb44) [14], *T. cruzi* flagellar calcium-binding protein (FCaBP) (Maric, *et. al.*, manuscript in preparation) and *Leishmania major* small myristoylated protein (SMP-1) [15]. Furthermore, lipid raft association is essential for the flagellar localization in the case of the *T. brucei* calflagins, where ablation of the palmitoylation site or inhibition of the enzyme that palmitoylates calflagin (TbPAT7) leads to protein mislocalization to the cell body membrane and disrupts its association with lipid rafts [16]. Hence it has been proposed that protein association with lipid rafts might serve to recruit and/or retain flagellar membrane proteins [14]. Despite these studies, it remains unclear how the organelle generates and maintains a lipid restrictive environment and the functional role of its membrane organization.

Protein composition of trypanosome flagellar membrane

In addition to unique lipid composition, flagellar membrane of trypanosomes is also characterized by the asymmetric distribution of certain proteins in comparison to other membrane domains. Proteins across the kinetoplastids that are heavily enriched or restricted to the flagellar membrane include: flagellar calcium-binding protein (FCaBP) (Figure 1 and [17]), calflagins (Tb 17, Tb24 and Tb44) [16], receptor adenylate cyclases (ESAG4) [18], low density lipoprotein receptor (LDL) [19], glucose transporter isoform 1 (Iso-1) [20] and the small myristoylated protein (SMP-1) [21]. A detailed listing of flagellar membrane proteins that have been confirmed in *Trypanosomes*, *Leishmania*, and *Chlamydomonas* is given in Table 1. The protein synthesis machinery is absent from the flagellum [22]; therefore, cells have evolved mechanisms to target newly synthesized flagellar proteins to their proper locations [1]. This is true for proteins of flagellar axoneme, flagellar matrix, and membrane, although the route of transport may differ among these classes of proteins. The mechanism by which proteins are selectively targeted to the flagellar membrane is somewhat controversial. The two prevailing models include: (i) a diffusion-retention model, in which proteins are delivered by a common pathway to the plasma membrane and can move laterally into the flagellar membrane and simply diffuse through the barrier imposed by the flagellar pocket and necklace, and (ii) a targeted delivery model, in which proteins

destined for either flagellar or cell body membranes are sorted at an earlier point in biosynthesis and are then carried in vesicles to the base of the flagellum to be separately delivered to their final destinations [23]. The evidence for the first model is for the most part based on the studies of flagellar attachment molecules called agglutinins in *Chlamydomonas* [24–26]. Flagellar agglutinins from the two gametes adhere to each other, enabling fertilization in *Chlamydomonas*. Upon attachment, the flagellar pool of active agglutinins is lost and is subsequently replaced by a pool from the cell body that is delivered by the lateral transport of the agglutinins from the plasma membrane to the flagellar membrane and doesn't involve vesicle transport to the base of the flagella or IFT [26,27]. More recently, Miletnkovic *et. al.* provided further evidence for lateral transport from the plasma membrane to the ciliary membrane in case of the ciliary protein Smoothened (Smo), an important protein in the Hedgehog signal transduction pathway. The binding of the ligand Sonic Hedgehog (Shh) to its receptor Patched 1 (Ptc1) serves as a trigger for lateral transport of Smo from the cell body membrane to the ciliary membrane and accumulation of Smo herein leads to the initiation of signaling [28]. Based on the work of others and recent findings from our own laboratory, it seems that the latter mechanism of protein delivery to the flagellar membrane is more likely in trypanosomes. Proteins that are part of the intraflagellar transport (IFT) machinery are synthesized in the cytoplasm on free polyribosomes and are imported post-translationally to the flagellum via the flagellar pore [4]. IFT protein complexes A and B move axonemal subunits to the tip of the flagellum (anterograde transport) and following cargo unloading at the flagellar tip, they return back to the base of the flagellum to be recycled (retrograde transport). Membrane-associated and transmembrane proteins are synthesized on the rough endoplasmatic reticulum (RER). Herein, proteins that need dual acylation, such as FCaBP/calflagins will get modified by the addition of a myristate to the N-terminal glycine residue by N-myristoyltransferase (NMT) and then will proceed to Golgi to get further modified via cysteine palmitoylation by one or more palmitoyltransferases (PAT). Golgi-derived vesicles differ in their lipid composition and this may in turn determine what kind of cargo they carry [29]. For example, vesicles that are enriched in sterols and sphingolipids may be preferentially loaded with lipid-modified proteins and proteins that contain flagellar targeting sequences. Additionally, since palmitoylation is reversible process, one or more of the PATs may also get loaded into these vesicles to confer association of their protein substrates to lipid raft-enriched vesicles. Vesicles of other composition may preferentially load other types of cargo, for example cargo destined for the cell body membrane. As the vesicles containing flagellum-bound cargo make their way towards the base of the flagellum, they interact with a multiprotein complex named BBSome [30,31] which facilitates transport of these vesicles to the base of the flagellum proximal to the flagellar pocket region [30,31]. Membrane vesicles cannot be targeted to the flagellar membrane directly and they accumulate at the base of the flagellum giving the appearance of a so called "flagellar necklace" structure [32]. At this point vesicles must fuse with the membrane, and in trypanosomes the fusion most likely occurs with the membrane of the flagellar pocket [26], a specialized invagination of the plasma membrane where all of the endo- and exocytosis takes place [33]. *Leishmania* glucose transporter isoforms 1 and 2 (Iso-1 and Iso-2) are both found in the flagellar pocket and are subsequently targeted to either the flagellar membrane (Iso-1) or to the plasma membrane (Iso-2) [20]. The differential localization of the two glucose transporter isoforms suggests that sorting takes place after the proteins reach the plasma membrane and that the flagellar pocket is the main sorting domain for membrane proteins in kinetoplastids [33]. Based on their intrinsic properties proteins get further sorted from the flagellar pocket to either flagellar or cell body membranes [33]. IFT proteins are likely also involved in the transport of the flagellar membrane proteins [1,34,35]. While the mode of protein entry into the flagella is controversial it is clear that flagellar membrane has a distinct protein composition in comparison to the flagellar pocket and cell body membranes. Presumably, the unique protein environment is what gives differential function to each membrane region.

Sensory and signaling functions of cilia in vertebrates

Both primary and motile cilia engage in signaling across diverse cells and tissues of multicellular eurkaryotes. In vertebrates, cilia transduce a variety of signals, including mechanical stressors from deformation or fluid flux (airway and kidney epithelia) [36,37], bending of otic hair cells in response to sound [38], others detect gradients for chemosensation [39], responsiveness to sex steroids [40] and light sensitivity by retinal photoreceptors [41]. The impact of ciliary function is equally diverse, ranging from tissue development via sensing of Hedgehog gradients [42] to the bulk movement of mucus during airway clearance. These processes generally result in signaling through alterations in cytoplasmic calcium mediated by voltage-sensitive channels, notably the transient receptor potential channels (TRP family), G-protein coupled receptor signaling, and/or activation of phospholipase C [43–45].

Sensory and signaling functions of cilia/flagella in invertebrates and protists

The evidence that both motile and nonmotile cilia have sensory and integrative signaling is not limited to vertebrates. Other eukaryotic organisms, notably *Caenorhabditis elegans* expresses the G-protein coupled receptor ODR-10 linked to ODR-3, which regulates a cation channel at the cilia of olfactory neurons [46–49]. Also in *C. elegans*, the TRP polycystin complex of proteins localizes to the distal ciliary tip and signals through phosphoinositides [50]. The protozoan *Paramecium* and green alga *Chlamydomonas* have several described integrative signaling pathways mediated by the flagellum. In *Paramecium*, a contact-sensitive process triggers calcium flux enabling a change in swimming trajectory [51], and sexual mating is initiated through cilium-cilium adhesion [52]. Examination of the detergent-resistant membranes of the *Chlamydomonas* flagellum revealed that light and oxidation state are sensed through a rhodopsin protein linking through flagellar AGG2/3 [53,54]. In the mating of *Chlamydomonas*, bidirectional flagellar contact initiates a signaling cascade through transient receptor potential (TRP) channels, involving a rise in both calcium and cyclic adenosine monophosphate (camp) [55]. These examples demonstrate that the varied and robust signaling role of cilia in vertebrate tissues is likely operative in many eukaryotic cells.

Sensory and signaling functions of trypanosome flagella

Although less well established, sensory and signaling roles for the trypanosome flagellum are highly predictable, given the structural similarities shared with other cilia. As discussed previously, the trypanosomal flagellar membrane is enriched in lipid rafts, platforms known to organize transmembrane signaling events [14]. In addition, many proteins, including several acylated proteins, are either enriched or are completely restricted to the flagellar membrane and many associate with lipid rafts [14,15 and Maric, et. al., manuscript in preparation]. Restricted localization of these molecules to the flagellar membrane suggests, but does not prove, specialization of their function. Several of these restricted proteins are predicted to be involved in sensing and signaling, extending the role of the trypanosome flagella beyond motility and host cell invasion. In motile cilia of protists and other invertabrates, such as *Chalmydomonas* and *Paramecium*, a rise in intracellular calcium and production of cAMPs in the cilia are known triggers that are important for sensory reception and initiation of downstream signaling events (reviewed in [56]). Similar themes are just beginning to emerge for the trypanosomatids. In *T. brucei*, the protein encoded by expression site associated gene 4 (ESAG4), an adenylyl cyclase, is restricted to the flagellar membrane and may mediate environmental sensing by regulating cAMP [18]. A flagellar cAMP signaling pathway in *T. brucei* through phosphodiesterases TbrPDEB1/B2 has been

implicated in parasite virulence [57], and a calcium handling protein calmodulin links to the paraflagellar rod [58]. The family of dually acylated flagellar calcium-binding proteins, *T. cruzi* FCaBP [59] and the *T. brucei* calflagins Tb17, Tb24, Tb44 [60] are all calcium sensors that associate with flagellar membrane in calcium-dependent and palmitoylation-dependent manner [17]. These proteins resemble the neuronal calcium sensors, which undergo a calcium-dependent conformational change that modulates the interaction between the acyl groups and the membrane [61]. This switch mechanism permits the association with two different binding partners, permitting an on/off switch to regulate their signaling functions [61]. Although the specific partner proteins of FCaBP/ calflagins are just now being identified, calcium-sensitive conformational changes have been confirmed [62]. Interestingly, a role for the calflagins in host survival and immune evasion has also recently been described [63]. Given the resurgence of interest in the cilia as a signaling platform linked to disease, that new proteins and signaling networks will be identified in the trypanosomal flagellum. We speculate that the calflagins will emerge as regulators of environmental sensing signaling through calcium and a regulated membrane association dependent upon their acylation state.

Conclusion

Cilia and flagella are fascinating organelles with a rich impact on tissue development, cell motility and environmental sensing. An improved understanding of ciliarly biology and has revealed several human disorders linked to defects in motility, IFT, and the BBsome complex. Beyond the common structural and motile elements that underlie most cilia, a new understanding of integrated environmental sensing is evident, and these recurring themes are likely extend to other protozoan organisms. Still, major questions remain about cilia and flagella: How is the specialized composition of the ciliary membrane established and then maintained? Where and how are cilium-bound vesicles formed and what are the precise molecular mechanisms of their transport and entry into the cilium? What is the molecular mechanism for each type of ciliopathy? What are the sensory functions of each type of cilium and the mechanisms by which the ciliary sensors transduce their signals into specific cellular responses? Finally, for digenetic organisms like trypanosomes, which spend part of their lives in invertebrate hosts and part in mammalian hosts (either intracellularly in *T. cruzi* and *Leishmania* species or extracellularly in *T. brucei*), what are the specific functions of the flagellum for adhesion to epithelial (insect) or endothelial (mammal) surfaces, cell invasion, differentiation and environmental sensing?

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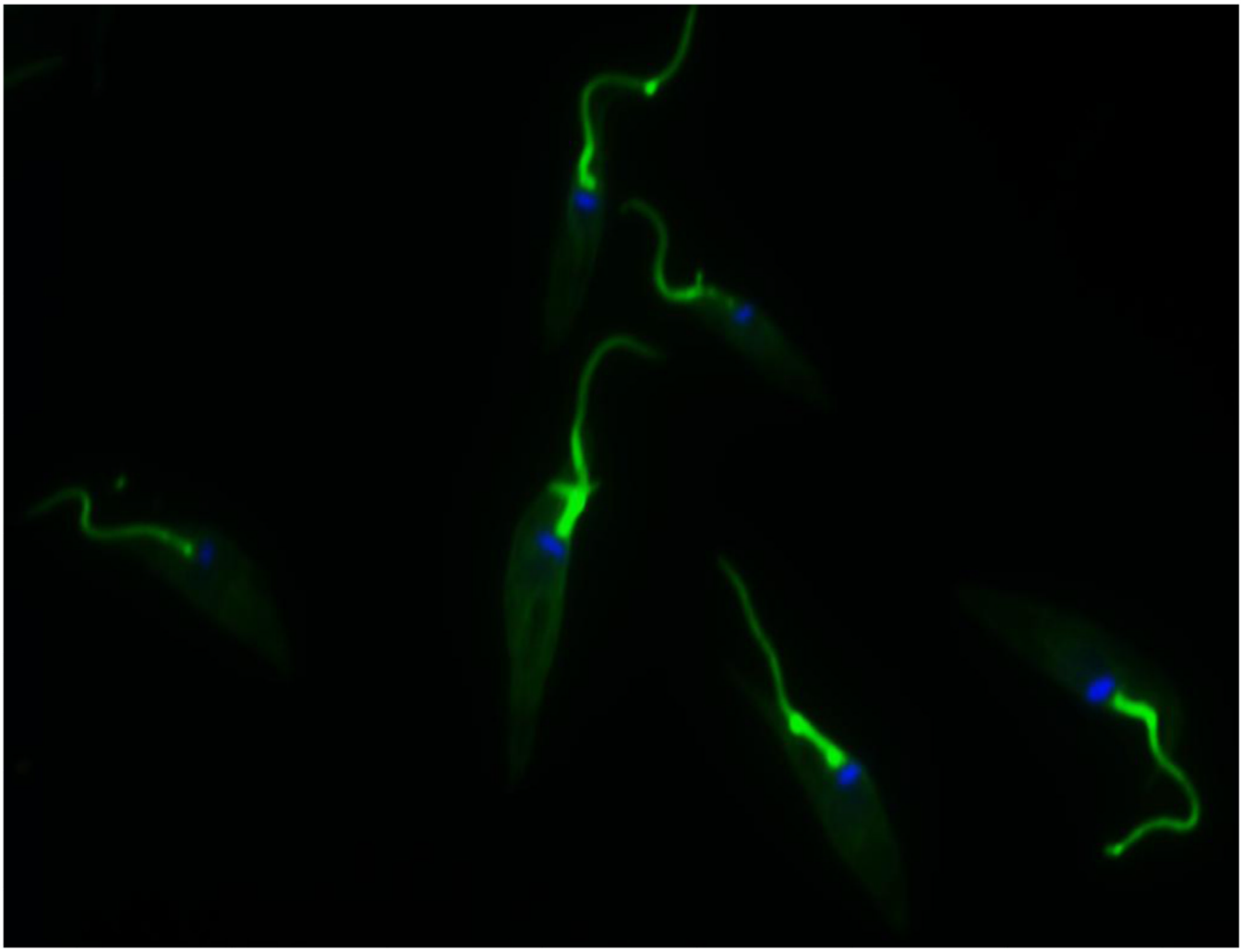

Figure 1.
The flagellar calcium-binding protein of *Trypanosoma cruzi*. FCaBP is a calcium sensor that localizes to the inner aspect of the flagellar membrane in a manner dependent on (1) dual acylation at the N-terminus by myristate and palmitate and (2) a high calcium concentration. Green: FCaBP immunofluorescence using a polyclonal FCaBP antibody. Blue: DAPI staining of parasite DNA (only the kinetoplast (mitochondrial) DNA) is clearly visible in this image.

Table 1

Inventory of confirmed flagellar membrane proteins in the trypanosomatids (*T. brucei*, *T. cruzi* and *Leishmania* spp.) and *Chlamydomonas reinhardtii*.

Protein	Organism	Reference
Calflagins Tb17, Tb24, Tb44	*Trypanosoma brucei*	[60,64]
Small kinetoplastid calpain-related proteins TbSKCRP7.2 and TbSKCRP1.5	*Trypanosoma brucei*	[65]
Glycosylphosphatidylinositol-specific phospholipase C, GPI-PLC	*Trypanosoma brucei*	[66]
Expression site associated gene 4, ESAG4	*Trypanosoma brucei*	[18]
Low density lipoprotein receptor, LDL	*Trypanosoma brucei*	[19]
Vacuolar type Ca^{2+} ATPase 1, TbA1 and TbA2	*Trypanosoma brucei*	[67]
Membrane-bound histidine acid phosphatase, TbMBAP1	*Trypanosoma brucei*	[68]
Flagellar calcium-binding protein, FCaBP	*Trypanosoma cruzi*	[69]
Small myristoylated protein-1, SMP-1	*Leishmania major*	[21]
Glucose transporter isoform-1, ISO-1	*Leishmania enriettii*	[20]
Glucose transporter 1, LmGT1	*Leishmania mexicana*	[70]
SST-2	*Leishmania braziliensis*	[71]
Aquaglyceroporin 1, AQP1	*Leishmania major*	[72]
Agg2p	*Chlamydomonas reinhardtii*	[54]
Kinesin-like protein FLA10	*Chlamydomonas reinhardtii*	[73]
Polycystin-2, TRPP2	*Chlamydomonas reinhardtii*	[74]
Polycystic kidney disease protein 1, PKD1	*Chlamydomonas reinhardtii*	[75]
Polycystic kidney disease protein 2, PKD2	*Chlamydomonas reinhardtii*	[76]
Agglutinins	*Chlamydomonas reinhardtii*	[77]
Flagella Membrane Glycoprotein 1B, FMG-1B	*Chlamydomonas reinhardtii*	[78,79]
Mastigoneme	*Chlamydomonas reinhardtii*	[79,80]